Detection, typing and control of *Histomonas meleagridis*

Harold van der Heijden
Utrecht
2009

Detection, typing and control of *Histomonas meleagridis*

Harold van der Heijden

ISBN: 978-90-393-49847
Omslag: Nanja Toebak
Lay-out: Multimedia, Diergeneeskunde, Universiteit Utrecht
Druk: Ridderprint, Ridderkerk
Uitgave: Gezondheidsdienst voor Dieren b.v.

Detection, typing and control of *Histomonas meleagridis*

Detectie, typering en controle van *Histomonas meleagridis*

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. J.C. Stoof,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op
donderdag 5 maart 2009 des middags te 12.45 uur

door

Henricus Marinus Johannes Franciscus van der Heijden

geboren op 18 december 1960 te Veghel

Promotor: Prof.dr. J.A. Stegeman

Co-promotor: Dr. W.J.M. Landman

Het in dit proefschrift beschreven onderzoek werd mede mogelijk gemaakt door financiële steun van: De Productschappen voor Vee, Vlees en Eieren en producenten van kalkoenvoer (in alfabetische volgorde): Agrifirm B.V., Boerenbond Deurne, Boerenbond Ysselsteyn Mengvoeders B.V., Brameco-Zon Voeders, Cehave Landbouwbelang Voeders B.V., Coöperatie Agruniek u.a., De Heus Voeders B.V., Isidorus Mengvoeders B.V. en Rijnvallei Diervoeder B.V.

Voor mijn moeder

Paranimfen: Hans van der Heijden
René Olthof

Dit is een uitgave van de Gezondheidsdienst voor Dieren b.v., Postbus 9, 7400 AA Deventer.

Contents

Chapter 1

General introduction

Historical perspectives

It is generally accepted that the first documented outbreak of histomonosis occurred by the end of the 19th century in Rhode Island, USA, although it was suggested (Tyzzer and Fabyan, 1920) that that the first allusion to this disease was made as long ago as 1674 (Josselyn, 1674). Until now it has always been assumed that the Rhode Island outbreak was first reported in 1893 (Cushman, 1893). However, historical research for this thesis revealed that already the 1891 annual report of the local Agricultural Experimental Station reported about an "obscure disease" observed for some years among turkeys, which was characterized by discolouration of the head and mortality, and was considered a threat to the turkey industry (Rice, 1892). The local farmers called the disease "blackhead" because at the height of the disease peculiar discolourations were observed in the head of some turkeys (Anonymous, 1894; Cushman, 1894, 1895). These discolourations refer to cyanosis of the head, which may or may not be present during disease. However, it is not a pathognomonic sign for histomonosis as it can be found in other disease conditions of turkeys. Since the disease was also associated with gross lesions of the ceca and liver, and a protozoan (*Amoeba meleagridis*) was identified as the probable causative organism, it was named infectious enterohepatitis (Smith, 1895). After its etiological agent was renamed to *Histomonas meleagridis* (Tyzzer, 1920a), the disease also became known as histomoniasis. An alternative term, histomonosis (Duffy et al., 2005; Hegngi et al., 1999; McGuire et al., 1963; Sullivan et al., 1965) has not been very popular until recent years. However, for reasons of standardization it was proposed (in 1990) to solely use the suffix -osis for parasitic diseases (Kassai, 2006) and therefore "histomonosis" has been adopted for this thesis.

Following the initial outbreak in Rhode Island, histomonosis rapidly spread through the USA and probably has caused more losses to the turkey production than any other single disease. The first confirmed case outside of the USA occurred in 1905 in Japan (Watanabé, 1910). Today, histomonosis has been reported worldwide (McDougald, 2005).

In the 1920's it was discovered that the cecal worm *Heterakis gallinarum* was a vector for the disease enabling the otherwise fragile *H. meleagridis* to be transmitted from one flock to the next (Graybill and Smith, 1920; Tyzzer, 1926). Additionally, chickens were identified as carriers of *H. meleagridis* and *H. gallinarum*, and as a source of infection to turkeys (Tyzzer, 1920b). Farmers were therefore advised to rear turkeys separately from fowl (Graybill and Smith, 1920).

The devastating consequences of the disease for commercial turkeys stimulated the analysis of many compounds for possible antihistomonal activity (McDougald, 2005) and both preventive and curative chemotherapeutics became readily available in the 1950's and 1960's. As a consequence, histomonosis in turkeys was rare during the 1970's and onwards.

Bringing the disease under control also represented a turning point in histomonas research: scientific interest in histomonosis was greatly reduced as demonstrated (Figure 1) by a dramatic decline in the number of scientific articles published on this matter (McDougald, 2007). However, due to increasing public concerns about food residues and the possible toxicity and carcinogenicity of the antihistomonal drugs, their use in production animals was banned some years ago (Byrne, 2001). This left the poultry industry and particularly the turkey farmer without effective treatment of histomonosis. Consequently, the disease re-emerged with up to 100% mortality in turkey flocks (Callait-Cardinal et al., 2007). Not unexpectedly, this was followed by a renewed interest in histomonosis by the scientific community as reflected by the number of scientific publications (Figure 1).

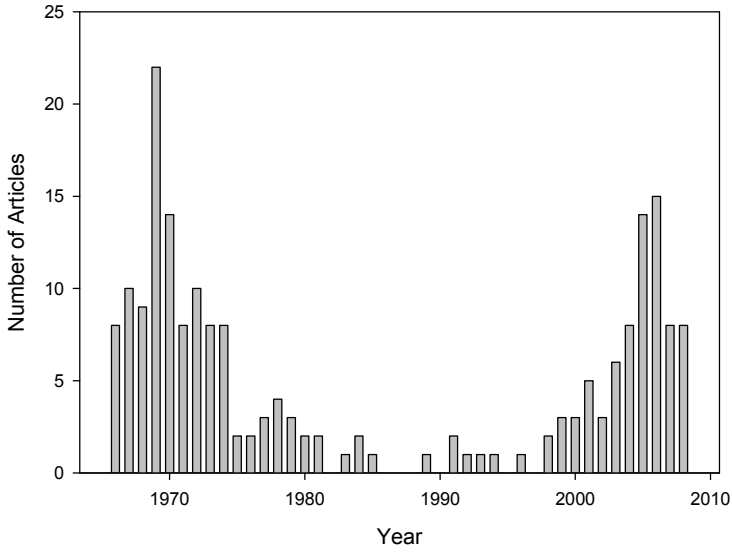


Figure 1: Number of scientific articles on *H. meleagridis* per year during the last four decades (kindly provided for this thesis by Prof. L. R. McDougald).

Histomonas meleagridis

The causative agent of histomonosis is a protozoan. Upon its discovery the organism was tentatively named *Amoeba meleagridis* (Smith, 1895; Tyzzer, 1919), but later the new genus *Histomonas* was proposed (Tyzzer, 1920a) as the parasite showed both amoebic and flagellar activity.

Two forms of the parasite are known: a tissue-dwelling form and a cecal lumen form. A cyst stage has not been convincingly identified so far. The tissue form is an almost round cell of 6-20 µm in size and is amoebic, forming pseudopodia at a temperature of 40 °C (McDougald and Reid, 1978). Unlike the tissue dwelling form the lumen form has a single flagellum although, early during cell division, two may be observed (Honigberg and Bennett, 1971). The beating of the flagellum causes a typical “rocking motion” of the cell at temperatures between 37 °C and 40 °C (Honigberg and Bennett, 1971), an observation that can help to distinguish *H. meleagridis* from other microorganisms with a similar morphology at light microscopy, e.g. *Blastocystis* spp. (McDougald, 2005; Tyzzer, 1919). The lumen dwelling form of *H. meleagridis* may be confused with another parabasalid species found in some common avian hosts (turkeys, chicken and pheasants): *Parahistomonas wenrichi* (Honigberg and Bennett, 1971; Honigberg and Kuldova, 1969; Lund, 1963; Wenrich, 1943). However, *Parahistomonas* has four instead of a single flagellum, is slightly larger than *H. meleagridis*, cannot be cultured in media in which *H. meleagridis* readily grows and is considered non-pathogenic.

The taxonomic classification of *H. meleagridis* is: phylum Parabasalia, class Trichomonadea, order Trichomonadidae and family of Monocercomonadidae (Adl et al., 2005). Parabasalia are anaerobic flagellated protists and have a parabasalid apparatus consisting of a parabasal body (Golgi-complex) and a parabasalid filament. These organisms possess hydrogenosomes (Mazet et al., 2008) rather than mitochondria and hence use an anaerobic metabolic pathway. *H. meleagridis* has a reduced cytoskeleton in the form of a micro tubular axostyle-pelta complex (Schuster, 1968) or a costa but without an undulating membrane (Mielewczik et al., 2008). One of the four basal bodies is located at the base of the flagellum (Mielewczik et al., 2008). *H. meleagridis* is both morphologically (Dobell, 1940; Schuster, 1968) and antigenically (Dwyer, 1972a, b, 1974) closely related to *Dientamoeba fragilis*, a human pathogen associated with gastrointestinal disorders (Johnson et al., 2004). Their relative taxonomic position was recently confirmed by a phylogenetic classification based on small subunit rRNA genes (Gerbod et al., 2001; Keeling, 2002).

Hosts

Histomonosis is mainly a disease of Galliform birds, turkeys being most susceptible with mortality percentages in flocks soaring up to 100% during outbreaks. In chickens, histomonosis was first reported in 1901 (Chester and Robin, 1901). Field reports document moderate mortality in this species of bird, which may linger for several weeks (McDougald, 2005). In young chickens, however, outbreaks with high mortality have been described. Chickens are considered to be an important

reservoir of the disease (Tyzzer, 1920b) and should be housed completely separate from highly susceptible birds (McDougald, 2005).

Many other species of Galliform and non-Galliform birds, e.g. peafowl, guinea fowl, pheasant, chuckar partridge, Japanese quail, bobwhite quail, ostrich, duck, goose, rhea, francolin and various species of zoo birds, were found to be infected with *H. meleagridis* with or without clinical signs (Borst and Lambers, 1985; Callait-Cardinal et al., 2006; Chute and Lund, 1972; Damodaran and Viswanathan, 1978; Desowitz, 1951; Dhillon, 1983; Douglass, 1981; Little et al., 1993; Lund and Chute, 1971, 1972a, b; Lund et al., 1974; Manuel and Guerzon, 1978; Mir et al., 1996; Wernery and Kinne, 2002; Zeakes et al., 1981). In some bird species mortality may be high, while in other species carriership of the parasite occurs.

Life Cycle and Epidemiology

H. meleagridis is a very fragile microorganism: naked histomonads, once excreted with faeces, can only survive for a short time at ambient temperature (McDougald, 2005). Strategically, the parasite uses an intermediate vector, the common cecal worm *Heterakis gallinarum* (Graybill and Smith, 1920; Tyzzer, 1926) to overcome this. In case of a co-infection *H. meleagridis* is able to invade the ova of *H. gallinarum*. Although it was suggested that the male heterakids become infected first and pass the histomonads to the female worms during copulation, this is not very likely as the stages of *H. meleagridis* among the sperm cells are too large to go directly to the unfertilized ova in the seminal receptacle (Lee, 1971). It seems therefore more probable that solely the female heterakid is responsible for the transmission of *H. meleagridis* to the worm egg (Lund and Chute, 1973). Inside the worm eggs, *H. meleagridis* is able to survive for a long time as was proven by inducing histomonosis in experimental poults that were fed with contaminated worm eggs that had been on the soil for as long as 150 weeks (Farr, 1961). Several species of earthworms, e.g. *Lumbricus terrestris* and *Eisenia* species were found to act as paratenic hosts for *H. gallinarum*, (Lund et al., 1966) by harbouring and concentrating *H. gallinarum* eggs and retaining hatched heterakis larvae (Augustine and Lund, 1974). A susceptible bird may become infected by both *H. gallinarum* and *H. meleagridis* by eating infected earthworms or by direct ingestion of contaminated *H. gallinarum* ova. Therefore, mechanical transport of cecal worm eggs, e.g. by personnel, visitors or animals, may be a relevant transmission route (McDougald, 2005). Some invertebrates like species of grasshoppers or flies have also been identified as (mechanical) vectors of cecal worm ova and even histomonosis (Frank, 1953).

The cecal worm as a vector is the most important transmission route of *H. meleagridis* between flocks or species of birds. However, recently, direct lateral transmission of *H. meleagridis*, likely occurring by “cloacal drinking” was

demonstrated in turkeys (Hu et al., 2004; Hu and McDougald, 2003; McDougald and Fuller, 2005) but not in chickens (Hu et al., 2006). It provides a plausible explanation for the rapid spread of the disease within commercial turkey flocks. Transmission of *Histomonas* by “cloacal drinking” may occur whenever the cloaca of a turkey comes into contact with infected fresh droppings. Histomonads may then be transferred into the cloaca by the cloacal reflex and into the ceca by reverse peristalsis (Hu et al., 2004).

Histomonosis

Disease occurs when histomonads penetrate the cecal wall, enter the blood stream and parasitize the liver. However, especially in less susceptible species of birds like chickens, pathological changes may remain confined to the ceca, the primary site of entry. Although the ceca and the liver are the main target organs for *H. meleagridis*, macro- and microscopical lesions and histomonal DNA detected by PCR or *in situ* hybridization have been found in several other organs following infection (Grabensteiner et al., 2006; Huber et al., 2006). In experimental infection using rectal inoculation of cultured histomonads, the incubation period is approximately 7 to 12 days. In case of oral inoculation with earth worms or *H. gallinarum* ova, cecal and liver lesions occur about three days later (McDougald et al., 1997).

Early clinical signs, although not pathognomonic but suggestive of histomonosis, are sulphur-coloured faeces, followed by drowsiness, dropping of wings, a stilted gait, closed eyes, head down and anorexia (Malewitz, 1956; McDougald et al., 1997).

Around 8 days following infection, primary lesions develop in the cecum. One or both ceca may be involved (Smith, 1895). After the cecal wall is invaded by histomonads, the ceca become thickened. A serous and hemorrhagic exudate from the cecal mucosa fills the lumen of the cecum. A dry caseous pale yellow core is formed from mucosa, serum proteins, blood and other debris (McDougald, 2005) and forces the cecal walls to distend. Ulceration of the cecal wall may lead to perforation resulting in peritonitis. Liver lesions, which appear around 10 days post infection, often are crater-like depressed areas with well-defined edges, but may also be small and numerous (McDougald, 2005; McDougald et al., 1997). Mortality may result from liver dysfunction or peritonitis, or both.

Laboratory tools

Diagnosis is usually based on gross pathology in combination with routine diagnostic histopathology after haematoxylin and eosin staining of cecal and liver

sections. In the centre of the liver lesions hepatocytes necrose and disintegrate. There is extensive lymphocytic and macrophage infiltration of the tissue. Histomonads occur individually or in clusters as round acidophilic forms (Brener et al., 2006).

For research purposes several other laboratory tools have been developed or applied, including culture (Drbohlav, 1924; Dwyer, 1970), electron microscopy (Schuster, 1968), Indirect Fluorescent Antibody Test (Augustine and Lund, 1970), Enzyme-linked ImmunoSorbent Assay (this thesis), Polymerase Chain Reaction (Huber et al., 2005), *in situ* hybridization (Liebhart et al., 2006) and genotyping (Van der Heijden et al., 2006).

Culture of protozoan parasites like *H. meleagridis* may be useful for (i) diagnosis, (ii) production of antigen, (iii) the identification of proteins involved in invasion, (iv) the assessment of functional antibodies, cell-mediated protective systems, lymphokines and other cytokines, (v) screening of chemotherapeutics, (vi) obtaining field isolates, (vii) the differentiation of susceptible from resistant isolates, (viii) the investigation of strain differences, (ix) the production of vaccines by attenuation, (x) the production of inocula for *in vivo* work, (xi) the study of their biochemistry and physiology, and (xii) the study of their morphology (Visvesvara and Garcia, 2002). However, culture of this type of organism is often cumbersome and laboratory workers have indicated that some “black magic” may be required for culture success (Visvesvara and Garcia, 2002).

Numerous culture media (Van der Heijden et al., 2005) for *H. meleagridis* have been tried, even some with “strange” additions like charcoal (Ruff and Hansen, 1970) or marmite (Berks and Neal, 1952). The first culture media successfully applied consisted of a combination of solid and liquid medium (Drbohlav, 1924), but later fully liquid media were developed (Devolt, 1943). These consisted of buffered saline supplemented with serum and other nutrient additions like rice starch (Bishop, 1938; Tyzzer, 1934). A frequently employed culture medium is Dwyer’s medium (Dwyer and Honigberg, 1970) which consists of Medium 199 with Hanks salts, 10% heat-inactivated horse serum, 5% chicken embryo extract and 10 to 12 mg of rice powder per 12.5 mL medium. Although many different media have been used, there are only a few studies reporting a systematic comparison of media or medium additions (Bishop, 1938; Devolt, 1943; Lesser, 1960, 1961; Stepkowski and Klimont, 1979). It is generally accepted that histomonads require co-cultivation with cecal bacteria (Stepkowski and Klimont, 1980) although some investigators have successfully used killed cecal bacteria (Lesser, 1961; Lund et al., 1967) instead of viable bacteria. There is consensus about the fact that anaerobic or microaerophilic conditions are required (Delappe, 1953).

It is possible to store *H. meleagridis* in liquid nitrogen and resuscitate the protozoan in culture or birds (Chute and Chute, 1969; Dwyer and Honigberg, 1970).

Antihistomonals

Many compounds have been used in the chemotherapy of histomonosis. Especially pentavalent arsenicals like nitarsone and nitroheterocylic compounds like dimetridazole proved highly effective against histomonosis (McDougald, 2005).

Nitarsone (4-nitrophenyl-arsonic acid) was widely used as a preventive feed additive (McGuire and Morehouse, 1952). The mechanism of action of arsenicals against various protozoa has not been fully elucidated, but they probably interfere with protozoan specific enzymes in hydrogenosomes and thiol metabolism (Krauth-Siegel and Coombs, 1999).

Dimetridazole is highly effective against histomonosis (Lucas, 1961) in all types of birds. It has been applied both as a preventive and as a curative chemotherapeutic (McDougald, 2005). Its mode of action is based on reduction of the nitro group when it interferes with the anaerobic metabolism of the protozoan. The resulting reactive metabolite interacts with DNA leading to subsequent inhibition of nucleic acid and protein synthesis (Callait et al., 2002; Moreno and Docampo, 1985).

In recent years in Europe all compounds which were known to be effective against histomonosis were banned because of increasing concern for the presence of residues in food and their possible toxic and carcinogenic properties (Anonymous, 1995, 2001; Byrne, 2001), while in the USA at present only nitarsone is permitted (McDougald, 2005). The fact that histomonosis re-emerged in the poultry industry stimulated research on alternative treatments including natural products (Zenner et al., 2003).

A cost-effective, animal-friendly means of testing new antihistomonals is an *in vitro* model (culture) rather than *in vivo* testing. However, it should be taken into account that both false-negative and false-positive results can be obtained using an *in vitro* test. Some products may only become active after metabolization in the host and will not show an *in vitro* inhibitory effect on *H. meleagridis*, while other products may inhibit only the bacteria that are needed by the protozoan when cultured (McDougald, 2005). Therefore, unfortunately, we cannot do without *in vivo* testing. Both chicken and turkey are readily infected following cloacal inoculation with a histomonad culture. After rectal inoculation birds should be kept in an inverted position for some minutes to prevent voiding of the inoculum (Chappel, 1975; Goedbloed and Bool, 1962).

Aim and objectives of this thesis

At the time this research project started (October 2003), no effective chemotherapeutics were available anymore to the poultry industry. Since the 1970's hardly any scientific studies on *H. meleagridis* or histomonosis had been

performed. This explains why modern laboratory tools like Enzyme-linked ImmunoSorbent Assay (ELISA), polymerase chain reaction (PCR) or DNA-sequencing had so far not been applied to this organism.

Therefore, the aim of this thesis was to optimize and develop new methods for the detection and typing of *H. meleagridis* and to examine new possibilities to control histomonosis in commercial poultry.

In Chapter one a general overview on *H. meleagridis* is given, including highlights in research (Table 1), description of the parasite and its hosts, its life cycle and epidemiology, the disease it causes, and its detection, typing and control.

In Chapter two an improvement of the culture medium for *Histomonas* is described, both with regard to the yield of parasites and the omission of chicken embryo extract from the medium. This work was done after the culture was standardized to give reproducible results, which is a prerequisite for reliable and cost-effective pre-screening of compounds with a possible antihistomonal effect.

Table 1: Highlights in *Histomonas* research.

Year	Reference	Highlight
1891	Rice (1892)	Histomonosis outbreak in Rhode Island, USA
1893	Cushman (1893)	"Blackhead" disease
1895	Smith (1895)	Etiology infectious enterohepatitis (*Amoeba meleagridis*)
1901	Chester & Robin	Infectious enterohepatitis in fowls
1905	Watanabé (1910)	Histomonosis outbreak outside USA
1920	Graybill & Smith (1920)	Transmission of protozoan by *H. gallinarum* ova
1919	Tyzzer (1920b)	Chicken as a source of infection for turkeys
1920	Tyzzer (1920a)	New genus *Histomonas*
1924	Drbohlav (1924)	Culture of *Histomonas meleagridis*
1940	Dobell (1940)	Comparison of *H. meleagridis* and *Dientamoeba fragilis*
1943	Wenrich (1943)	*Parahistomons wenrichi*
1951	McGuire & Morehouse (1952)	Nitarsone is a prophylactic chemotherapeutic
1961	Lucas (1961)	Dimetridazole is an effective chemotherapeutic
1966	Lund et al (1966)	Earthworm as paratenic vector
1969	Chute & Chute (1969)	Cryopreservation
1970	Dwyer (1970)	Dwyers culture medium
2001	Anonymous (2001)	Ban on dimetridazoles and arsenical therapeutics
2002	Hu & McDougald (2003)	Direct transmission through cloacal drinking
2003	Zenner et al (2003)	Antihistomonal effect of natural products
2004	Huber et al (2005)	PCR
2006	Hess et al (2006)	Clonal culture
2006	Van der Heijden et al (2006)	Molecular typing by C-profiling of ITS1 sequences
2008	Hess et al (2008)	Effective vaccination
2008	Van der Heijden et al (this thesis)	ELISA

In Chapter three the standardized culture and an animal model that was developed were used to test several herbal products and other compounds for their antihistomonal efficacy. This concerned several feed additives that were being marketed as antihistomonals with no or hardly any scientific proof for this claim.

In Chapter four the development and validation of an ELISA for *H. meleagridis* serology is described. It is a valuable addition to the available laboratory tools if it can be used to perform epidemiological studies in commercial poultry in a reliable and inexpensive way. Moreover, it could represent a sensitive monitoring technique in transmission studies.

In Chapter five the development of a novel genetic typing method for *H. meleagridis* is outlined. Our understanding on the spread and transmission of *H. meleagridis* may further increase as isolates from different sources and outbreaks are genotyped. Genotyping will be especially useful if different genotypes can be linked to differences in virulence. Further, genetic typing may also shed some light on the phylogenetic position of non-pathogenic histomonas-like protozoa such as *P. wenrichi*.

This thesis is finalized with a summarizing discussion.

References

Anonymous 1894. Work in the poultry division. In: Ninth Annual Report of the State Board of Agriculture, p. 109.

Anonymous 1995. Annex IV of council regulation EEC No. 1798/95.

Anonymous 2001. Council regulation (EC) No. 1756/2002.

Augustine, P.C., Lund, E.E., 1970, Indirect fluorescent antibody tests comparing two strains of *Histomonas meleagridis* and *Histomonas wenrichi*. Journal of Protozoology 17, 97-99.

Augustine, P.C., Lund, E.E., 1974, The fate of eggs and larvae of *Ascaridia galli* in earthworms. Avian Diseases 18, 394-398.

Berks, G., Neal, R.A., 1952, The effect of some drugs upon *Histomonas meleagridis in vitro*. Annals of Tropical Medicine and Parasitology 46, 68-71.

Bishop, A., 1938, *Histomonas meleagridis* in domestic fowls (*Gallus gallus*). cultivation and experimental infection. Parasitology 30, 181-194.

Borst, G.H.A., Lambers, G.M., 1985, Typhlohepatitis in ostriches (*Struthio camelus*) caused by *Histomonas* infection. Tijdschrift Voor Diergeneeskunde 110, 536-537.

Brener, B., Tortelly, R., Menezes, R.C., Muniz-Pereira, L.C., Pinto, R.M., 2006, Prevalence and pathology of the nematode *Heterakis gallinarum*, the trematode *Paratanaisia bragai*, and the protozoan *Histomonas meleagridis* in the turkey, *Meleagris gallopavo*. Memorias Do Instituto Oswaldo Cruz 101, 677-681.

Byrne, D., 2001, Commission regulation EC No. 2205/2001, amending council directive 70/524/EEC concerning additives in feedings stuffs as regards Wwthdrawal of the authorization of certain additives. Official Journal of the European Community L297, 3-4.

Callait-Cardinal, M.P., Chauve, C., Reynaud, M.C., Alogninouwa, T., Zenner, L., 2006, Infectivity of *Histomonas meleagridis* in ducks. Avian Pathology 35, 109-116.

Callait-Cardinal, M.P., Leroux, S., Venereau, E., Chauve, C.M., Le Pottier, G., Zenner, L., 2007, Incidence of histomonosis in turkeys in France since the bans of dimetridazole and nifursol. Veterinary Record 161, 581-585.

Callait, M.P., Granier, C., Chauve, C., Zenner, L., 2002, *In vitro* activity of therapeutic drugs against *Histomonas meleagridis* (Smith, 1895). Poultry Science 81, 1122-1127.

Chappel, L.R., 1975, Using Hml1 strain of *Histomonas meleagridis* in evaluating chemotherapeutants in chicks. Avian Diseases 19, 679-682.
Chester, F.D., Robin, M.D. 1901. Entero-hepatitis or blackhead of fowls. In: 12th Annual Report of Delaware College Agricultural Experimental Station, pp. 60-66.
Chute, A.M., Lund, E.E., 1972, Experimental gistomoniasis in guinea fowl, *Numida meleagris*. Journal of Protozoology 19, 639-643.
Chute, M.B., Chute, A.M., 1969, Freeze preservation of *Histomonas meleagridis*. Poultry Science 48, 2189-2191.
Cushman, S., 1893, The production of turkeys. Rhode Island State Bulletin, 89-123.
Cushman, S., 1894. A study of the diseases of turkeys. In Sixth Annual Report of the Rhode Island Agricultural Experiment Station 1893, pp. 286-288.
Cushman, S., 1895, An infectious disease of turkeys. In: Seventh Annual Report of the Rhode Island Agricultural Experimental Station 1894. pp. 199-201.
Damodaran, S., Viswanathan, S., 1978, Histomoniasis in a pea fowl (*Pavo cristata*). Indian Veterinary Journal 55, 922-922.
Delappe, I.P., 1953, Studies on *Histomonas meleagridis*. III. The influence of anaerobic versus aerobic environments on the growth of the organism *in vitro*. Experimental Parasitology 2, 209-222.
Desowitz, R.S., 1951, Age as a factor influencing fatal infections of histomoniasis in chickens. Journal of Comparative Pathology and Therapeutics 61, 231-236.
Devolt, H.M., 1943, A new medium for the cultivation of *Histomonas meleagridis*. Journal of Parasitology 29, 353-355.
Dhillon, A.S., 1983, Histomoniasis in a captive great rhea (*Rhea americana*). Journal of Wildlife Diseases 19, 274-274.
Dobell, C., 1940, Researches on the intestinal protozoa of monkeys and man. X. The life history of *Dientamoeba fragilis*: observations, experiments, and speculations. Parasitology 32, 417-461.
Douglass, E.M., 1981, Histomoniasis in zoo birds. Veterinary Medicine & Small Animal Clinician 76, 1013-1014.
Drbohlav, J., 1924, The cultivation of the protozoon of blackhead. Journal of Medicinal Chemistry 44, 677-678.
Duffy, C.F., Sims, M.D., Power, R.F., 2005, Evaluation of dietary natustat for control of *Histomonas meleagridis* in male turkeys on infected litter. Avian Diseases 49, 423-425.
Dwyer, D.M., 1970, An improved method for cultivating *Histomonas meleagridis*. Journal of Parasitology 56, 191-192.
Dwyer, D.M., Honigberg, B.M., 1970, Effect of certain laboratory procedures on virulence of *Histomonas meleagridis* for turkeys and chickens. Journal of Parasitology 56, 694-700.
Farr, M.M., 1961, Further observations on survival of protozoan parasite, *Histomonas meleagridis*, and eggs of poultry nematodes in feces of infected birds. Cornell Veterinarian 51, 3-13.
Frank, J.F., 1953, A note on the experimental transmission of enterohepatitis of turkeys by arthropods. Canadian Journal of Comparative Medicine 17, 230-231.
Gerbod, D., Edgcomb, V.P., Noel, C., Zenner, L., Wintjens, R., Delgado-Viscogliosi, P., Holder, M.E., Sogin, M.L., Viscogliosi, E., 2001, Phylogenetic position of the trichomonad parasite of turkeys, *Histomonas meleagridis* (Smith) Tyzzer, inferred from small subunit rRNA sequence. Journal of Eukaryotic Microbiology 48, 498-504.
Goedbloed, E., Bool, P.H., 1962, Protozoan etiology of blackhead. Avian Diseases 6, 302-315.
Graybill, H.W., Smith, T., 1920, Production of fatal blackhead in turkeys by feeding embryonated eggs of *Heterakis Papillosa*. Journal of Experimental Medicine 31, 647-655.
Hegngi, F.N., Doerr, J., Cummings, T.S., Schwartz, R.D., Saunders, G., Zajac, A., Larsen, C.T., Pierson, F.W., 1999, The effectiveness of benzimidazole derivatives for the treatment and prevention of histomonosis (Blackhead) in turkeys. Veterinary Parasitology 81, 29-37.
Hess, M., Kolbe, T., Grabensteiner, E., Prosl, H., 2006, Clonal cultures of *Histomonas meleagridis*, *Tetratrichomonas gallinarum* and a *Blasctocystis* sp. established through micromanipulation. Parasitology 133, 547-554.
Hess, M., Liebhart, E., Grabensteiner, E., Singh, A., 2008, Cloned *Histomonas meleagridis* passaged *in vitro* resulted in reduced pathogenicity and is capable of protecting turkeys from histomonosis. Vaccine 26, 4187-4193.

Honigberg, B.M., Bennett, C.J., 1971, Lightmicroscopic observations on structure and division of *Histomonas meleagridis* (Smith). Journal of Protozoology 18, 687-697.
Hu, J., Fuller, L., Armstrong, P.L., McDougald, L.R., 2006, *Histomonas meleagridis* in chickens: attempted transmission in the absence of vectors. Avian Diseases 50, 277-279.
Hu, J., Fuller, L., McDougald, L.R., 2004, Infection of turkeys with *Histomonas meleagridis* by the cloacal drop method. Avian Diseases 48, 746-750.
Hu, J.H., McDougald, L.R., 2003, Direct lateral transmission of *Histomonas meleagridis* in turkeys. Avian Diseases 47, 489-492.
Huber, K., Chauve, C., Zenner, L., 2005, Detection of *Histomonas meleagridis* in turkeys cecal droppings by PCR amplification of the small subunit ribosomal DNA sequence. Veterinary Parasitology 131, 311-316.
Johnson, E.H., Windsor, J.J., Clark, C.G., 2004, Emerging from obscurity: biological, clinical, and diagnostic aspects of *Dientamoeba fragilis*. Clinical Microbiology Reviews 17, 553-570.
Josselyn, J., 1674, Josselyn's voyages - An account of two voyages to New England, London.
Kassai, T., 2006, Nomenclature for parasitic diseases: cohabitation with inconsistency for how long and why? Veterinary Parasitology 138, 169-178.
Keeling, P.J., 2002, Molecular phylogenetic position of *Trichomitopsis termopsidis* (parabasalia) and evidence for the trichomitopsiinae. European Journal of Protistology 38, 279-286.
Krauth-Siegel, R.L., Coombs, G.H., 1999, Enzymes of parasite thiol metabolism as drug targets. Parasitology Today 15, 404-409.
Lee, D.L., 1971, Structure and development of protozoon *Histomonas meleagridis* in male reproductive tract of its intermediate host, *Heterakis gallinarum* (Nematoda). Parasitology 63, 439-445.
Lesser, E., 1960, Cultivation of *Histomonas meleagridis* in a modified tissue culture medium. Journal of Parasitology 46, 686-686.
Lesser, E., 1961, *In vitro* cultivation of *Histomonas meleagridis* free of demonstrable bacteria. Journal of Protozoology 8, 228.
Liebhart, D., Weissenbock, H., Hess, M., 2006, *In situ* hybridization for the detection and identification of *Histomonas meleagridis* in tissues. Journal of Comparative Pathology 135, 237-242.
Little, R.M., Earle, R.A., Crowe, T.M., Huchzermeyer, F.W., 1993, Mortality caused by histomoniasis in young greywing francolin. South African Journal of Wildlife Research 23, 57-58.
Lucas, J.M.S., 1961, 1,2-Dimethyl-5-nitroimidazole, 8595 R.P. Part I. Prophylactic activity against experimental histomoniasis in turkeys. Veterinary Record, 465-467.
Lund, E.E., Augustine, P.C., Chute, A.M., 1967, *Histomonas meleagridis* after one thousand *in vitro* passages. Journal of Protozoology 14, 349-351.
Lund, E.E., Chute, A.M., 1971, Histomoniasis in chukar partridge. Journal of Wildlife Management 35, 307-315.
Lund, E.E., Chute, A.M., 1972a, Reciprocal responses of 8 species of galliform birds and 3 parasites - *Heterakis gallinarum*, *Histomonas meleagridis*, and *Parahistomonas wenrichi*. Journal of Parasitology 58, 940-945.
Lund, E.E., Chute, A.M., 1972b, The ring-necked pheasant (*Phasianus colchicus* Torquatus) as a host for *Heterakis gallinarum* and *Histomonas meleagridis*. American Midland Naturalist 87, 1-7.
Lund, E.E., Chute, A.M., 1973, Means of acquisition of *Histomonas meleagridis* by eggs of *Heterakis gallinarum*. Parasitology 66, 335-342.
Lund, E.E., Chute, A.M., Vernon, M.E.L., 1974, Experimental infections with *Histomonas meleagridis* and *Heterakis gallinarum* in ducks and geese. Journal of Parasitology 60, 683-686.
Lund, E.E., Wehr, E.E., Elli, D.J., 1966, Earthworm transmission of *Heterakis* and *Histomonas* to turkeys and chickens. Journal of Parasitology 52, 899-902.
Malewitz, T.D., 1956, Studies on the pathology of experimentally produced blackhead in turkeys. Journal of Parasitology 42, 23-23.
Manuel, M.F., Guerzon, V.A., 1978, Survey on the occurrence of *Histomonas meleagridis* in locally raised domestic quails (*Coturnix coturnix* japonicum). Philippine Journal of Veterinary Medicine 17, 121-129.
Mazet, M., Diogon, M., Alderete, J.F., Vivares, C.P., Delbac, F., 2008, First molecular characterisation of hydrogenosomes in the protozoan parasite *Histomonas meleagridis*. International Journal for Parasitology 38, 177-190.

McDougald, L.R., 2005, Blackhead disease (histomoniasis) in poultry: a critical review. Avian Diseases 49, 462-476.
McDougald, L.R., 2007. Histomoniasis in poultry: An overview of recent research. In: International Symposium on Protozoal Infections in Poultry, Vienna, Austra, p. 6.
McDougald, L.R., Calneck, B., Barnes, H.J., Beard, C.W., Saif, Y.M., 1997, Other protozoan diseases of the intestinal tract - histomoniasis (blackhead), In: Disease of Poultry. Iowa State University Press, Ames, pp. 1001-1006.
McDougald, L.R., Fuller, L., 2005, Blackhead disease in turkeys: Direct transmission of *Histomonas meleagridis* from bird to bird in a laboratory model. Avian Diseases 49, 328-331.
McDougald, L.R., Reid, W.M., 1978, *Histomonas meleagridi*s and relatives. In: Parasitic Protozoa. pp. 139-161.
McGuire, W.C., Moeller, M.W., Morehouse, N.F., 1963, Effect of dimetridazole on growth and prevention of histomonosis in poultry. Poultry Science 42, 1289.
McGuire, W.C., Morehouse, N.F., 1952, Chemotherapy studies of histomoniasis. Poultry Science 31, 603-609.
Mielewczik, M., Mehlhorn, H., Al-Quraishy, S., Grabensteiner, E., Hess, M., 2008, Transmission electron microscopic studies of stages of *Histomonas meleagridis* from clonal cultures. Parasitology Research 103, 745-750.
Mir, A.S., Shahardar, R.A., Pandit, B.A., Ahmed, M.A., 1996, Occurrence of histomoniasis (enterohepatitis) in hen reared partridges (*Gracia electoris*) in Kashmir. Indian Veterinary Journal 73, 98-99.
Moreno, S.N.J., Docampo, R., 1985, Mechanism of toxicity of nitro compounds used in the chemotherapy of trichomoniasis. Enviromental Health Perspectives, 64: 199-208.
Rice, F.E., 1892. Disease among turkeys. In Fourth Annual Report of the State Board of Agriculture p. 96.
Ruff, M.D., Hansen, M.F., 1970, *Histomonas meleagridis* - effect of gamma radiation on *in vitro* growth. Experimental Parasitology 28, 450-464.
Schuster, F.L., 1968, Ultrastructure of *Histomonas meleagridis* (Smith) Tyzzer, a parasitic ameboflagellate. Journal of Parasitology 54, 725-737.
Smith, T., 1895, An infectious disease among turkeys caused by protozoa (infectious entero-hepatitis). Bulletin of the United States Department of Agriculture 8, 7-38.
Stepkowski, S., Klimont, S., 1979, Observations on the culture of *Histomonas meleagridis* (Smith 1895) *in vitro*. Medycyna Weterynaryjna 35, 502-505.
Stepkowski, S., Klimont, S., 1980, The influence of some microorganisms on the *in vitro* multiplication of *Histomonas meleagridis*. Wiad Parazytol 26, 635-643.
Sullivan, T.W., Whitmore, J.H., Grace, O.D., Kingan, J.R., 1965, Effect of certain blackhead preventive compounds on growth and their efficacy against histomonosis in turkeys. Poultry Science 44, 1420.
Tyzzer, E.E., 1919, Developmental phases in the protozoon of blackhead in turkeys. Journal of Medical Research 40, 1-32.
Tyzzer, E.E., 1920a, The flagellate character and reclassification of the parasite producing "blackhead" in turkeys - *Histomonas* (gen. nov.) *meleagridis* (Smith). Journal of Parasitology 6, 124-131.
Tyzzer, E.E., 1920b, Observations on the transmission of blackhead in turkeys - the common fowl as a source of infection. Journal of Medical Research 4, 219-237.
Tyzzer, E.E., 1926, *Heterakis vesicularis* Frölich 1791: A vector of an infectious disease. Proceedings of the Society for Experimental Biology and Medicine 23, 708-709.
Tyzzer, E.E., 1934, Studies of histomoniasis, or "blackhead" infection, in the chickens and the turkey. Proceedings of the American Academy of Arts and Sciences 69, 189-264.
Tyzzer, E.E., Fabyan, M., 1920, Further studies on "blackhead" in turkeys, with special reference to transmission by inoculation. Journal of Infectious Diseases 27, 207-329.
Van der Heijden, H.M., Landman, W.J., Greve, S., Peek, R., 2006, Genotyping of *Histomonas meleagridis* isolates based on internal transcribed spacer-1 sequences. Avian Pathology 35, 330-334.
Van der Heijden, H.M., McDougald, L.R., Landman, W.J., 2005, High yield of parasites and prolonged *in vitro* culture of *Histomonas meleagridis*. Avian Pathology 34, 505-508.

Visvesvara, G.S., Garcia, L.S., 2002, Culture of protozoan parasites. Clinical Microbiology reviews 15, 327-328.

Watanabé, Y., 1910, On the so-called black-head of turkey. Transactions of the Sapporo Natural and Historical Society, 67-80

Wenrich, D.H., 1943, Observations on the morphology of *Histomonas* (Protozoa, Mastigophora) from pheasants and chickens. Journal of Morphology 72, 279-304.

Wernery, U., Kinne, J., 2002, Blackhead in Arabian red-legged partridge, stone Curlew and spotted thick-knee (dikkop). Falco 19, 19.

Zeakes, S.J., Hansen, M.F., Robel, R.J., 1981, Increased susceptibility of bobwhites (*Colinus virginianus*) to *Histomonas meleagridis* after exposure to sevin insecticide. Avian Diseases 25, 981-987.

Zenner, L., Callait, M.P., Granier, C., Chauve, C., 2003, *In vitro* effect of essential oils from *Cinnamomum aromaticum*, *Citrus limon* and *Allium sativum* on two intestinal flagellates of poultry, *Tetratrichomonas gallinarum* and *Histomonas meleagridis*. Parasite 10, 153-157.

Chapter 2

In vitro culture of *Histomonas meleagridis*

Chapter *2.1*

High yield of parasites and prolonged *in vitro* culture of *Histomonas meleagridis*

H.M.J.F. van der Heijden[1], L.R. McDougald[2] and W.J.M. Landman[1]

1. Animal Health Service Ltd, P.O. Box 9, 7400 AA Deventer, the Netherlands
2. Department of Poultry Science, University of Georgia, USA

Published: Avian Pathology, 34 (2005) 505-508

Abstract

Dwyer's medium is a frequently employed culture medium for *Histomonas meleagridis* with rice powder as an essential ingredient. The effect of adding larger quantities of rice powder to the culture medium and the influence of the size of the rice particles on the growth of *H. meleagridis* was examined. Increasing the amount of rice powder from the standard amount of 10 to 12 mg to 50 to 100 mg per 12.5 ml medium resulted in approximately a 10-fold increase of parasites. Larger quantities of rice powder did not give better yields. The particle size of the rice powder proved relatively unimportant, although the addition of only large rice powder particles (> 250 µm) resulted in a somewhat lesser yield. *H. meleagridis* cultures could be prolonged from approximately 4 days to at least 2 weeks without subculturing by supplementing the culture medium with rice powder only.

Introduction

The protozoan parasite *Histomonas meleagridis* (Tyzzer and Fabyan, 1920) causes histomonosis or blackhead disease (Smith, 1895) in turkeys, chicken and other species of gallinaceous birds. In the past decades, treatment and control of histomonosis was possible using a number of drugs including arsenicals and nitroimidazoles (Hegngi et al., 1999). Increasing concern about drug residues in food and the identification of some compounds as potentially carcinogenic has led to a ban of these chemotherapeutics within the European Community (Zenner et al., 2003). Developing possible alternative intervention measures such as vaccination and treatments with natural plant extracts for anti-Histomonas activity (Zenner et al., 2003) requires diagnostic tools, such as an *in vitro* assay (Callait et al., 2002) or an enzyme-linked immunosorbent assay. In order to develop these tools, it was necessary to improve the currently available culturing method for *H. meleagridis*, especially increasing the yield of parasites to provide for enzyme-linked immunosorbent assay antigen production.

After the first successful attempt to culture the protozoan *in vitro* (Drbohlav, 1924), numerous culture media have been described. Initially a combination of a solid and a liquid medium was attempted (Drbohlav, 1924; Tyzzer, 1936), but later completely liquid culture media were developed (DeVolt, 1943). These usually consisted of buffered saline with 5 to 10% serum (of various mammal species) and other additions, including rice powder (Tyzzer, 1934, 1936; Bishop, 1938; DeVolt, 1943; Delappe 1953; McDougald and Reid, 1978), marmite (Lucas and Goose, 1965), turkey caecal bacteria (Lesser, 1960, 1964; Lund et al., 1967), cream (Lesser, 1963, 1964), granular charcoal (Ruff and Hansen, 1970; Hirsch and Hirsch, 1977) and chicken embryo extract (Dwyer, 1970; Stepkowski and Klimont, 1979). A frequently used culture medium is Medium 199 with Hank's salts, 10% heat-inactivated horse serum, 5% chicken embryo extract and 10 to 12 mg rice powder per 12.5 ml medium (Dwyer, 1970; McDougald and Reid, 1978). The amount of rice powder is considered critical as addition of larger or smaller amounts resulted in decreased yield of histomonads (Dwyer, 1970). However, no data supporting this observation have been published, and no other factors such as particle size of the ground rice powder were considered. In the present study the positive effect of a higher amount of rice powder in Dwyer's culture medium on *H. meleagridis* growth *in vitro* is demonstrated.

Materials and Methods

***H. meleagridis* strain, culture medium and long-term storage**. A field strain of *H. meleagridis*, strain AL237, was isolated from the caeca of turkeys presenting liver and caecal lesions by culturing in Dwyer's culture medium (Dwyer, 1970). This

culture medium consisted of 85% Medium 199 with Hank's salts (Gibco-BRL, Grand Island, New York, USA), 10% heat-inactivated horse serum (Gibco-BRL), 5% chicken embryo extract (8-day-old embryos were homogenized in a blender, freeze-thawed once and stored at -20 °C until used) and 10 to 12 mg white rice powder (Arrowhead Mills Inc., Melville, New York, USA). Caeca were opened using scissors and the fluid between the inflammatory core and intestinal wall was collected using a sterile Pasteur pipette and inoculated into 12.5 ml culture medium in 70 ml tissue culture flasks (Becton Dickinson, France). The culture was incubated at 40 °C for 48 h before subculturing 1 ml into 12.5 ml freshly prepared culture medium. Growth of histomonads was assessed daily by counting the number of parasites per millilitre using a Bürker-Türk haematocytometer and a microscope at 200x magnification. For long-term storage, 10% dimethylsulfoxide (Merck, Germany) was added as a cryoprotectant (Dwyer and Honigberg, 1970). The suspension was aliquoted into vials of 1 ml and frozen to -70 °C under controlled conditions (approximately -1 °C/min). Vials were then stored in liquid nitrogen (-175 °C) until use. The viability of isolates stored under these conditions was repeatedly confirmed by *in vitro* culture and by experimental infection of turkeys resulting in histomonosis (Landman et al., 2004). The *H. meleagridis* strain used in the present study was subcultured once, stored in liquid nitrogen, resuscitated and subcultured once more (counts, $10^{5.6}$ histomonads/ml) before further storage in liquid nitrogen.

Amount of rice powder. A vial with 1 ml *H. meleagridis* strain stored in liquid nitrogen was thawed quickly in room-temperature water. The whole volume was then inoculated into 12.5 ml freshly prepared and pre-warmed Dwyer's culture medium with 12.0 mg rice powder. After incubation for 72 h at 40 °C, tissue culture flasks with 12.5 ml Dwyer's medium containing different amounts of rice powder were inoculated with 1 ml *H. meleagridis* culture (approximately $10^{5.7}$ histomonads). Several experiments with either 0, 10, 20, 40, 60, 80, 100, 150 or 200 mg (± 0.1 mg) rice powder per 12.5 ml Dwyer's medium were performed. Negative control cultures were included. Growth of histomonads was monitored by counting the number of parasites using a Bürker-Türk haematocytometer (magnification 200x) each day at around 08:00, 12:00 and 16:00 h. The cultures were also examined microscopically daily at a higher magnification (400x) to ascertain identification of the parasite (e.g. flagellum, typical ''rocking'' motion). Every 2 days (approximately 48 h) the cultures were supplemented with (only) the same amount of rice powder as was used initially (i.e. no subculturing).

Rice powder particle size. To examine the effect of the particle size on histomonad culturing, the rice powder was sieved through two filters: a 0.25 mm (NEN2560 checked) steel wire sieve (Metaalgaasweverij, Twente, The Netherlands) and a 100 µm nylon sieve (Stokvis and Smits, The Netherlands). This

resulted in rice powder with different particle sizes: standard (not sieved), > 250 µm, < 250 µm, 100 to 250 µm, and < 100 µm. Culture medium was prepared with either 12 or 100 mg rice powder of these five different particle sizes and inoculated with *H. meleagridis* as already described.

Results

Amount of rice powder. Without supplementation of the medium with rice powder (Figure 1), the concentration of histomonads in culture increased to approximately 10^5 histomonads/ml in 2 days, after which the concentration dropped below the detection limit (10^4/ml) within the next 2 days. However, when cultures were supplemented with rice powder (10 mg per culture flask into 12.5 ml culture) every 48 h, the concentration of histomonads could be maintained at approximately 10^6/ml for over 300 h (12 days). This was followed by a decrease in the histomonad concentration in the next 200 h (until day 21), after which viable organisms could still be observed.

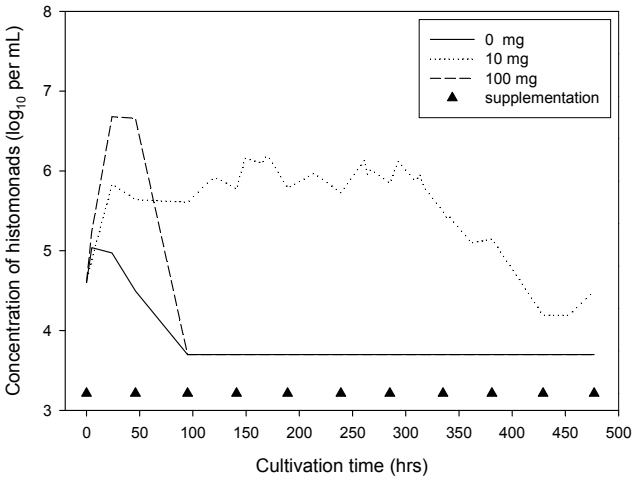


Figure 1: Prolonged cultivation of *Histomonas meleagridis* without sub-culturing by regular supplementation of culture medium with 0, 10 or 100 mg rice powder per flask (12.5 ml). The detection limit is $10^{3.7}$ histomonads/ml.

If larger amounts of rice powder were used instead of 10 to 12 mg (e.g. 100 mg; see Figure 1), the concentration of histomonads became considerably higher (more than 10^7/ml) within 2 days, but then supplementation did not prevent the cultures

from dying out within 4 days. This experiment was repeated, with almost identical results (the concentration of histomonads in cultures supplemented with 10 mg rice powder started to drop after 250 h of incubation instead of 300 h). In negative control cultures, the parasite counts were always negative.

The increase of histomonad concentration in cultures using higher amounts of rice powder than 10 to 12 mg was examined in two similar experiments where amounts up to 100 or 200 mg rice powder per 12.5 ml culture medium were used. In Figure 2 the histomonad concentrations are shown for the experiment using cultures with 0, 10, 20, 40, 60, 80, 100, 150, or 200 mg rice powder per 12.5 ml medium determined at 4, 8, 24, 32, or 49 h after start of the culture. The results of the other experiment (rice powder amounts up to 100 mg) were similar (data not shown). Between 24 and 32 h after incubation, the histomonad concentration reached a maximum of around 10^7 histomonads/ml when approximately 50 to 100 mg rice powder was added to the medium. This concentration of histomonads was up to 10 times higher than the concentration when a standard amount of 10 mg rice powder was used. Greater amounts of rice powder either did not result in higher concentrations of histomonads (150 mg) or resulted in somehwat lower histomonad concentrations (200 mg).

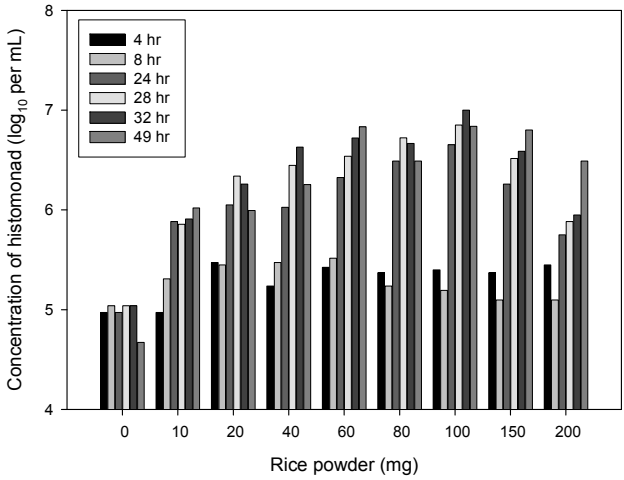
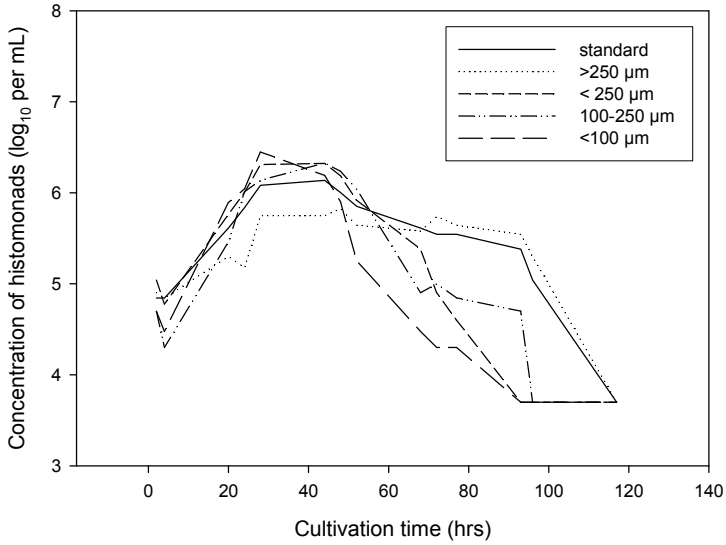


Figure 2: The effect of increasing amounts of rice powder in the culture medium on the concentration of histomonads in cultures of different ages.

A



B

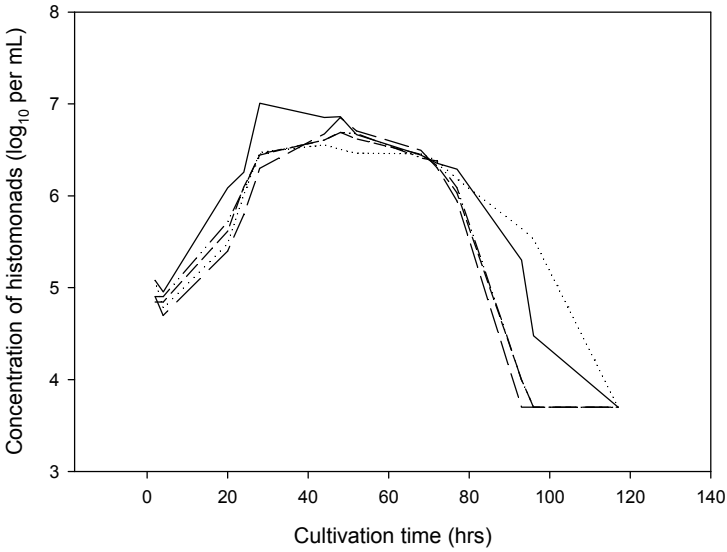


Figure 3: The effect of particle size of rice powder on the histomonad concentrations determined at different incubation times (no supplementation). 3a: 12 mg of rice powder added, 3b: 100 mg of rice powder added.

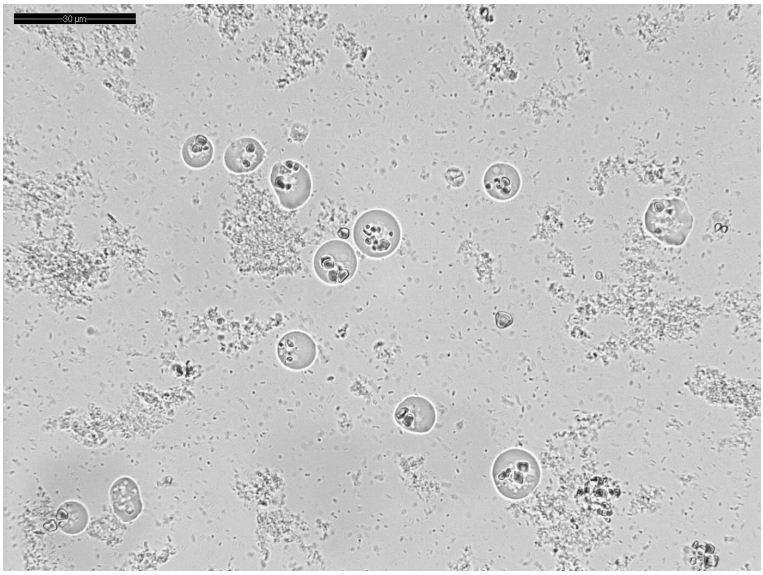


Figure 4: *Histomonas meleagridis* in dixenic culture (note phagocytized rice particles).

Rice powder particle size. Standard rice powder consisted of 74% particles > 250 µm, 15% of particles sized between 100 and 250 µm, and 11% of particles smaller than 100 µm. The effect of different rice particle sizes on the histomonad concentrations found during culturing (see Figure 3a,b) was relatively small in comparison with the effect of the amount of rice powder. Only the cultures with the largest particle sizes (> 250 µm) tended to yield lower maximum histomonad concentrations. It appeared that histomonads only ingested small rice particles as all rice granules observed inside histomonads were < 5 µm (Figure 4).

Discussion

Rice powder is an essential constituent of Dwyer's culture medium (Dwyer, 1970). When rice powder was omitted from the medium, no multiplication of histomonads was observed (McDougald and Reid, 1978). Histomonads probably feed directly on the rice powder, since many of the parasites show rice particle inclusions at microscopy (Tyzzer, 1934; Lucas and Goose, 1965) but the rice powder probably also provides food for specific bacteria that serve as food for histomonads (Tyzzer, 1934; McDougald and Reid, 1978). These bacteria are coisolated with the

Histomonas strains from the caeca of turkeys or chickens and are essential for multiplication of the histomonads (Lund et al., 1967; Berks and Neal, 1952; Lesser, 1960; Augustine and Chute, 1978). Other ingredients of Dwyer's medium are horse serum and chicken embryo extract (Dwyer, 1970).

Typically, *H. meleagridis* reach maximum numbers *in vitro* at 2 or 3 days after inoculation (Dwyer, 1970). Populations of histomonads decrease rapidly and die out after approximately 5 days, probably due to depletion of nutrients. Tyzzer (1934) mentioned that the life of a culture could be prolonged by adding small amounts of rice powder at regular intervals, without giving further details. In the present study, we found that *H. meleagridis* cultures remain viable for at least 21 days without subculturing when only a small amount (10 to 12 mg) of rice powder was added. We also observed that, even if the medium still contained visible quantities of rice powder, the numbers of parasites declined. In addition it was observed that ingested rice particles were always approximately uniform in size. Our hypothesis is that histomonads can only directly ingest very small (< 5 µm) rice powder particles, and that large rice powder particles are only indirectly a source of nutrients through the action of bacteria. Attempts to culture *H. meleagridis* using rice powder as the only nutrient added to M199 medium (i.e. without serum and chicken extract), with 1% gentamycin added to kill the bacterial flora, were not successful (data not shown).

Traditionally 10 to 15 mg ground rice powder, or one ground rice grain per culture tube or flask, with 10 to 15 ml Dwyer's medium has been used (Dwyer, 1970; Hirsch and Hirsch, 1977; McDougald and Reid, 1978). Dwyer (1970) explicitly stated that the amount of rice powder was critical (i.e. larger or smaller amounts resulted in decreased yield of parasites). However, there are no data supporting this observation, nor has there been systematic examination by other workers.

Typically, concentrations ranging from $10^{5.5}$ to 10^{6} histomonads/ml have been found as maximum yields (Delappe 1953; Hirsch and Hirsch, 1977; Stepkowski and Klimont, 1979). The same yields were also obtained in the present study when 10 to 12 mg rice powder was used. However, 50 to 100 mg rice powder per 12.5 ml medium gave significantly higher yields of parasites, concentrations sometimes exceeding 10^{7} parasites/ml. Greater amounts of rice powder were not beneficial and it was not possible to extend the life of *H. meleagridis* cultures based on more than 12 mg rice powder by adding extra rice powder at regular intervals. Such cultures died out rapidly, and no organisms were found anymore. The histomonads grew abundantly in the first 2 days, and probably nutrients other than rice powder quickly became exhausted. When the counting interval is too long, it is possible to miss the abundant growth phase and increased yield (Figure 1). This might be an explanation for Dwyer's recommendation that no more than 12 mg rice powder should be used (Dwyer, 1970).

The influence of rice powder particle size on the maximum yield of histomonads proved relatively unimportant. When only large rice powder particles (> 250 µm) were used (which made up 75% of the standard rice powder), the yield of parasites was slightly less. In some studies (Tyzzer, 1934; Hirsch and Hirsch, 1977), finely ground rice powder was preferred over coarser grades, but others successfully used whole solid rice grains instead (Bishop, 1938). The size of the rice particles depends on the way the rice is ground and probably varies between different brands, and possibly between different lots. Although the present study seems to indicate that particle size was relatively unimportant, it could be worth considering when culturing of *H. meleagridis* proves difficult or results in poor yields.

References

Augustine, P.C., Chute, A.M., 1978, *In vitro*-adapted *Histomonas meleagridis* in gnotobiotic turkeys. Journal of Parasitology 64, 425.

Berks, G., Neal, R.A., 1952, The effect of some drugs upon *Histomonas meleagridis in vitro*. Annals of Tropical Medicine and Parasitology 46, 68-71.

Bishop, A., 1938, *Histomonas meleagridis* in domestic fowls (*Gallus gallus*). Cultivation and experimental infection. Parasitology 30, 181-194.

Callait, M.P., Granier, C., Chauve, C., Zenner, L, 2002, *In vitro* activity of therapeutic drugs against *Histomonas meleagridis* (Smith, 1895). Poultry Science 81, 1122-1127.

Delappe, I.P., 1953, Studies on *Histomonas meleagridis* - II. Influence of age of original inoculum and pH on growth in various media. Experimental Parasitology 2, 117-124.

DeVolt, H.M., 1943, A new medium for the cultivation of *Histomonas meleagridis*. Journal of Parasitology 29, 353-355.

Drbohlav, J., 1924, The cultivation of the protozoön of blackhead. Journal of Medical Research 44, 677-678.

Dwyer, D.M., 1970, An improved medium for cultivating *Histomonas meleagridis*. Journal of Parasitology 56, 191-192.

Dwyer, D.M., Honigberg, B.M., 1970, Effect of certain laboratory procedures on the virulence of *Histomonas meleagridis* for turkeys and chickens. Journal of Parasitology 56, 694-700.

Hegngi, F.N., Doerr, J., Cummings, T.S., Schwartz, R.D., Saunders, G., Zajaz, A., Larsen, C.T., Pierson, F.W., 1999, The effectiveness of benzimidazole derivatives for the treatment and prevention of histomoniasis (blackhead) in turkeys. Veterinary Parasitology 81, 29-37.

Hirsch, R.P., Hirsch, D.S., 1977, Methods of sterilizing DeVolt's medium for *in vitro* culture of *Histomonas meleagridis*. Journal of Parasitology 63, 386-388.

Landman, W.J.M., McDougald, L.R., Van der Heijden, H.M.J.F., 2004, Experimental infestation of turkeys and chickens with a Dutch field isolate of *Histomonas meleagridis*. In H.M. Hafez (Ed.), Proceedings of the 5th International Symposium on Turkey Diseases (pp. 53-54). Berlin, Germany.

Lesser, E., 1960, Replacement of serum in the cultivation of *Histomonas meleagridis*. Journal of Parasitology 46, 271.

Lesser, E., 1963, Effect of amphotericin-B on *in vitro* growth of *Histomonas meleagridis*. Journal of Parasitology 49, 329.

Lesser, E., 1964, Studies on the *in vitro* cultivation of *Histomonas meleagridis* with three different mixed bacterial flora. Proceedings of the Helminthological Society of Washington 31, 263-264.

Lucas, J.M.S., Goose, J., 1965, Tests for activity against *Histomonas meleagridis*: use of the infection in chickens and a comparison of the results obtained in chickens and turkeys. British Veterinary Journal, 121, 65-75.

Lund, E.E., Augustine, P.C., Chute, A.M., 1967, *Histomonas meleagridis* after one thousand passages. Journal of Protozoology 14, 349-351.

McDougald, L.R., Reid, W.M., 1978, *Histomonas meleagridis* and relatives. In J.P. Kreier (Ed.), Parasitic Protozoa, Vol. II (pp. 139-161). New York: Academic Press.

Ruff, M.D., Hansen, M.F., 1970, *Histomonas meleagridis*: effect of gamma radiation on *in vitro* growth. Experimental Parasitology 28, 450-464.

Smith, T., 1895, An infectious disease among turkeys caused by the protozoa (infectious entero-hepatitis). US Department of Agriculture Bulletin 8, 7-90.

Stepkowski, S., Klimont, S., 1979, Obserwacje nad hodowla *in vitro Histomonas meleagridis*. Medycyna Weteryanaryjna 35, 502-505.

Tyzzer, E., 1934, Studies on histomoniasis or ‘‘blackhead’’ infection, in the chicken and the turkey. Proceedings of the American Academy of Arts and Sciences 69, 189-264.

Tyzzer, E., 1936, A study of immunity by infection with attenuated culture-strains of *Histomonas meleagridis*. The Journal of Comparative Pathology and Therapeutics 49, 285-303.

Tyzzer, E.E., Fabyan, M., 1920, Further studies on ‘‘blackhead’’ in turkeys, with special reference to transmission by inoculation. Journal of Infectious Diseases 27, 207-239.

Zenner, L., Callait, M.P., Granier, C., Chauve, C, 2003, *In vitro* effect of essential oils from *Cinnamomum aromatocum*, *Citrus limon* and *Allium savitum* on two intestinal flagellates of poultry, *Tetratrichimonas gallinarum* and *Histomonas meleagridis*. Parasite 10, 153-157.

Chapter 2.2

Improved culture of *Histomonas meleagridis* in a modification of Dwyer medium

H.M.J.F. van der Heijden and W.J.M. Landman

Animal Health Service Ltd, P.O. Box 9, 7400 AA Deventer, the Netherlands

Published: Avian Diseases, 51 (2007) 986-988

Summary

Dwyer medium is the most frequently employed culture medium for *Histomonas meleagridis*. Both for subculturing and for resuscitation of *H. meleagridis* from storage in liquid nitrogen, modified Dwyer medium with an increased rice powder concentration (0.8%) and no chicken embryo extract proved superior to Dwyer standard medium, with threefold ($10^{6.3}$ vs. $10^{5.8}$ histomonads/ml) to 10-fold ($10^{6.7}$ vs. $10^{5.8}$ histomonads/ml) higher concentrations of parasites after resuscitation or subculturing, respectively.

Introduction

Histomonas meleagridis may cause histomonosis, or blackhead disease, in gallinaceous birds. In turkey flocks mortality can be as high as 85% (McDougald, 2005). Although numerous media have been attempted for cultivation of this protozoan (Van der Heijden et al., 2005), during the last 3 decades Dwyer medium (Dwyer, 1970) has been almost exclusively used. Its constituents are Medium 199 (M199) with Hank's salts, horse serum, chicken embryo extract (CEE), and rice powder. In addition, *H. meleagridis* usually only grows readily in dixenic cultures, i.e., with fecal bacteria coisolated with the parasite (Landman et al., 2004). In order to more efficiently produce *H. meleagridis* antigen for enzyme-linked immunosorbent assay and other purposes, recently the yield of parasites after culture was greatly improved by increasing the amount of rice powder in the medium from 0.096% (w/v) to 0.8% (w/v) (Van der Heijden et al., 2005). Purification of histomonads still proved difficult because of the complex composition of Dwyer culture medium. Therefore, in the present study it was examined whether the CEE or horse serum could be omitted from the culture medium used for subculturing of histomonads. In addition it was tested whether the medium without CEE was also suited for recovery of histomonads from liquid nitrogen (resuscitation).

Materials and Methods

Media. Standard Dwyer medium (Dwyer, 1970) for cultivation of *H. meleagridi*s consisted of 85% M199 with Hank's salts (Gibco-BRL, Grand Island, NY), 10% heat-inactivated horse serum (Gibco-BRL), 5% CEE homogenate (8-day-old chicken embryos were homogenized in a blender, freeze-thawed once, and stored at -20 °C until used), and 12 mg of white rice powder (Arrowhead Mills Inc., Hereford, TX) per 12.5 ml (0.096% w/v). High-yield medium (Van der Heijden et al., 2005) had an increased rice powder concentration (0.8%) in comparison with standard Dwyer medium. In the present study a modified Dwyer medium was developed that consisted of M199 with 0.8% (w/v) rice powder, 10% horse serum and no CEE (i.e., high-yield medium without CEE). In addition, leaving horse serum out of the culture medium was attempted (Table 1).

***Histomonas meleagridis* strain**. Isolate Turkey/NL/Dev/AL327-type I/03 (Van der Heijden et al., 2005) was used for all experiments. The isolate was stored in 1 ml aliquots ($10^{5.6}$ histomonads) in liquid nitrogen as described elsewhere (Van der Heijden et al., 2005) and successfully resuscitated on numerous occasions as confirmed by *in vitro* culture and experimental inoculation of turkeys resulting in histomonosis (Landman et al., 2004).

Table 1. Overview of experiments and details on changes in constituents of Dwyer standard culture medium for *H. meleagridis*

		medium components			medium
experiment	purpose	CEE	serum	rice	name
1	subculture	5%	10%	0.8%	high yield
		0%	10%	0.8%	modified
2	subculture	5%	10%	0.8%	high yield
		0%	10%	0.8%	modified
		0%	0%	0.8%	no serum
3	subculture	5%	10%	0.096%	standard
		no	10%	0.8%	modified
4	resuscitation	5%	10%	0.096%	standard
		no	10%	0.8%	modified

Experiments. In the first three experiments the *H. meleagridis* isolate was resuscitated in standard Dwyer medium. Subculturing was done after 72 h by inoculating 1 ml of culture into 12.5 ml of fresh medium. In the first experiment subculturing was performed using either high yield medium or modified Dwyer medium. In the second experiment, high yield medium, modified Dwyer medium, or modified Dwyer medium without horse serum was used for subculturing. In the third experiment subculturing was done either in standard Dwyer medium or in modified Dwyer medium. In the fourth experiment, the isolate was resuscitated in standard medium, subcultured in modified Dwyer medium, and 20 aliquots of 1.0 ml were stored in liquid nitrogen. One week later five aliquots were resuscitated in standard Dwyer medium and five aliquots were resuscitated in modified Dwyer medium. This experiment was repeated one month after storage.

In all experiments, growth of parasites was monitored by counting the number of parasites using a Bürker-Türk hematocytometer (magnification 200x) at regular intervals (usually three times per day) with a detection limit of $10^{3.7}$ histomonads/ml.

Results

In the first experiment (Figure 1) no differences in the numbers of parasites were observed when *H. meleagridis* was subcultured in high yield medium or modified Dwyer medium. In all cases the concentration of histomonads increased from 10^5 histomonads/ml to $10^{6.7}$ histomonads/ml within 30 h, after which the numbers of

parasites no longer increased. Similar results were observed when the CEE extract was clarified from cellular debris by centrifugation before addition to the medium (data not shown).

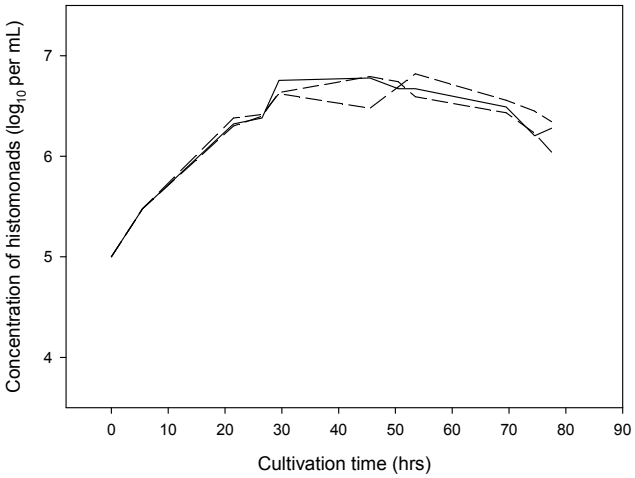


Figure 1. Subculturing of *H. meleagridis* in either high yield medium (continuous line), or modified Dwyer's medium (duplicate; dashed lines).

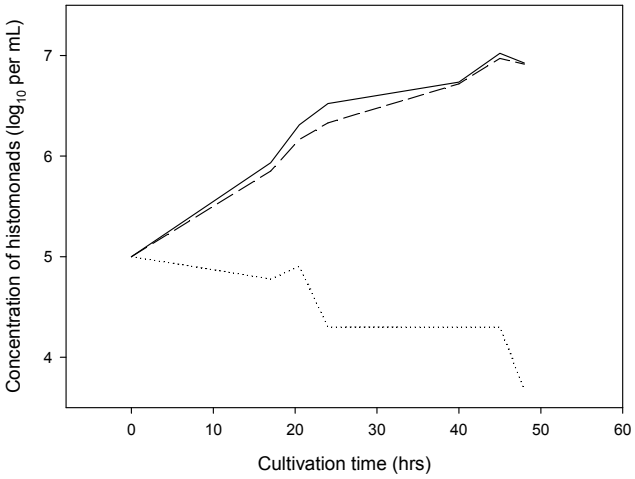


Figure 2. Subculturing of *H. meleagridis* in either high yield medium (continuous line), modified Dwyer's medium (dashed line), or modified Dwyer's medium without horse serum (dotted line).

In the second experiment, again comparing high-yield medium with modified Dwyer medium for subculturing, the results were comparable to the first experiment (Figure 2). The concentrations of parasites increased from 10^5 histomonads/ml to $10^{6.7}$ histomonads/ml within 40 h. In contrast, when horse serum was left out of modified Dwyer medium the numbers of parasites declined during culturing (Figure 2).

In the third experiment, a direct comparison of standard and modified Dwyer medium for subculturing, after 24 h clearly higher numbers of parasites were already obtained with modified Dwyer medium (Figure 3). After 2 days the difference in the concentration of histomonads between the two media was almost 10-fold ($10^{6.7}$ histomonads/ml vs. $10^{5.8}$ histomonads/ml).

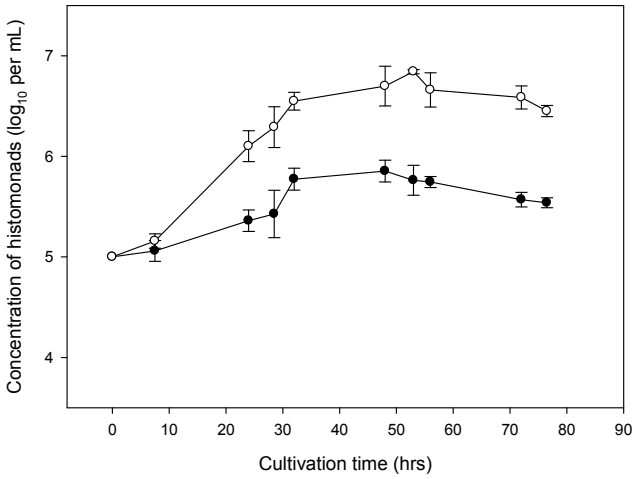


Figure 3. Subculturing of *H. meleagridis* in standard Dwyer medium (black dots) or modified Dwyer medium (white dots). Average results and error bars indicating standard deviations of pentaplate cultures.

In the fourth experiment, *H. meleagridis* was resuscitated using either standard Dwyer medium or modified Dwyer medium. During the first 32 h after resuscitation the results obtained with the two media were similar (Figure 4). After 2 days and during the rest of the experiment the histomonad counts were on average $10^{5.8}$ histomonads/ml when standard Dwyer medium was used for resuscitation and $10^{6.3}$ histomonads/ml when modified Dwyer medium was used. This experiment was repeated one month after storage with almost identical results.

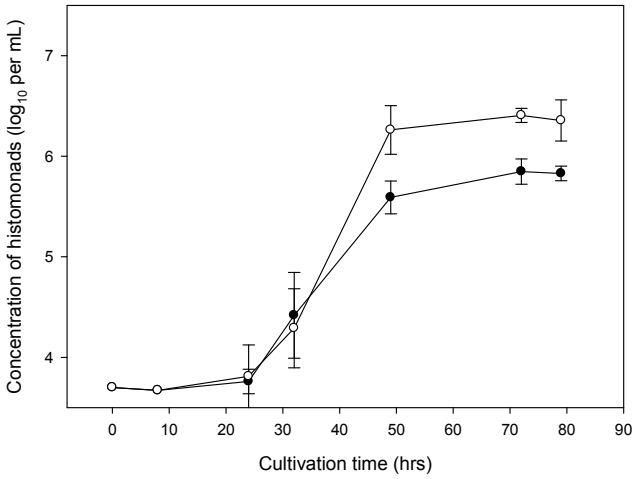


Figure 4. Resuscitation of *H. meleagridis* in standard Dwyer medium (black) or modified Dwyer medium (white dots). Average results and error bars indicating standard deviations of pentaplate cultures.

Discussion

CEE homogenate is a complex component of standard Dwyer medium that may vary in composition between production lots. Because of tissue and cell remainders, the culture becomes cloudy and eventually contains aggregates. This makes it difficult to isolate histomonads from culture, e.g., for production of antigen. Until recently, the necessity for using CEE and other culture constituents had not been systematically examined.

Stepkowksi–Klimont medium (Stepkowski and Klimont, 1979) is very similar to Dwyer medium. The difference is that the former is based on Eagle salts rather than Hank's salts. Recently it was also mentioned that for Stepkowski–Klimont medium CEE was dispensable (Hess et al., 2006), though no further details were provided.

A recently proposed modification of Dwyer standard medium was to use 0.8% of rice powder (high-yield medium) instead of 0.096%. This greatly improved the growth and yield of parasites (Van der Heijden et al., 2005). The present study shows that using modified Dwyer medium (i.e., with 0.8% rice powder but without CEE) still resulted in approximately 10-fold higher concentrations of histomonads in comparison with standard Dwyer medium. On the other hand, it was demonstrated that horse serum is an essential ingredient since without it no growth of histomonads was seen, and eventually the cultures died out.

In addition, it was tested whether modified Dwyer medium was also suitable for recovering the parasites from storage in liquid nitrogen, because it could well have been possible that this requires a richer medium. Nevertheless, after resuscitation the parasites grew equally well in modified and standard Dwyer medium. Following successful recovery, after 2 days the parasite counts for the modified medium became threefold higher, which was to be expected from the results found in the subculturing experiments.

The modified Dwyer medium is an improvement over Dwyer standard medium and high-yield medium for resuscitation and subculturing of *H. meleagridis*, because it combines higher yields of parasites (Van der Heijden et al., 2005) with a simplification of the medium composition. To further simplify the culture medium for histomonads in order to be able to more easily obtain pure histomonal antigens, the need for dixenic culture should be reexamined. Conflicting reports have been published, including reports of axenic *H. meleagridis* culture with antibiotics added to culture medium, sterile culture media with heat-killed bacteria as an essential ingredient, and dixenic culture (McDougald, 2005).

References

Dwyer, D.M., 1970, An improved medium for cultivating *Histomonas meleagridis*. Journal of Parasitology 56,191–192.

Hess, M., Kolbe, T., Grabensteiner, E., Prosl, H., 2006, Clonal cultures of *Histomonas meleagridis*, *Tetratrichomonas gallinarum* and a *Blastocystis* sp. established through micromanipulation. Parasitology 133, 547–554.

Landman, W.J.M., McDougald, L.R., Van der Heijden, H.M.J.F., 2004, Experimental infestation of turkeys and chickens with a Dutch field isolate of *Histomonas meleagridis*. In H.M. Hafez (Ed.), Proceedings of the 5th International Symposium on Turkey Diseases, Berlin, Germany, pp. 53-54.

McDougald, L.R., 2005, Blackhead disease (histomoniasis) in poultry: a critical review. Avian Diseases 49, 462–476.

Stepkowski, S., Klimont, S., 1979, Obserwacje nad hodowla *in vitro Histomonas meleagridis*, Medycyna Weteryanaryjna 35, 502–505.

Van der Heijden, H.M.J.F., McDougald, L.R., Landman, W.J.M., 2005, High yield of parasites and prolonged *in vitro* culture of *Histomonas meleagridis*. Avian Pathology 34, 505–508.

Chapter 3

In vitro and *in vivo* efficacy of herbal products and antibiotics against *Histomonas meleagridis*

Chapter 3.1

In vitro effect of herbal products against *Histomonas meleagridis*

H.M.J.F. van der Heijden and W.J.M. Landman

Animal Health Service Ltd, P.O. Box 9, 7400 AA Deventer, the Netherlands

Published: Veterinary Parasitology, 154 (2008) 1-7

Abstract

The ban on chemotherapeutics against *Histomonas meleagridis* in the European Union has left turkey producers without an effective treatment against histomonosis. It has encouraged the development of alternative control methods amongst which are a number of herbal products. In the present study the *in vitro* effect of four herbal products against *H. meleagridis* was examined. After suspension of the herbal products in Dwyer's culture medium used for subculturing of a viable culture, parasite growth was monitored by frequently counting the histomonads until 72 h of incubation. The solid products Aromabiotic™, Enteroguard™, and Protophyt SP™ as well as the positive control products dimetridazole and Histostat-50™, were tested in twofold serial dilutions ranging in concentration from 400 to 12.5 µg/ml, while the liquid product Protophyt B™ was tested in concentrations ranging from 0.24% to 0.008% (v/v). The herbal products Enteroguard™ and Protophyt B™ as well as dimetridazole and Histostat-50™, two chemotherapeutics with known antihistomonal activity, inhibited the growth of *H. meleagridis*, while no antihistomonal effect was found for Aromabiotic™ and Protophyt SP™.

Introduction

Histomonas meleagridis is an amoeboid protozoan with a single flagellum and may cause histomonosis (infectious enterohepatitis; blackhead disease) in galliforms and other species of birds. The disease can result in high mortality in turkeys, while in chicken histomonosis is generally less severe (McDougald, 1997). However, chicken flocks may form a disease reservoir with the cecal worm *Heterakis gallinarum* acting as an intermediate vector between chicken and turkey flocks. The very fragile *H. meleagridis* protozoa may infect heterakid eggs (Graybill and Smith, 1920) and thus remain infective in soil or faeces for years (McDougald, 2005).

During the last decades several effective chemotherapeutics like nifursol, arsenical compounds (e.g., nitarsone and carbasone), some nitrofurans, and nitroimidazoles (e.g., dimetridazole, ipronidazole, and ronidazole) were available as preventive feed additives or as curative therapeutics (Hegngi et al., 1999; McDougald, 2005). However, increasing concern for the presence of residues in food and possible toxicity and carcinogeneticy of these therapeutics led to their ban in Europe some years ago, while in the US at present only nitarsone is permitted (Byrne, 2001; Zenner et al., 2003; McDougald, 2005).

In recent studies several compounds and products were found to have an antihistomonal activity. These included nitroimidazoles, arsenicals (Callait et al., 2002; Hu and McDougald, 2004), volatile oils of *Cinnamomum aromaticum*, *Citrus limon*, and *Allium sativum* (Zenner et al., 2003), a plant-derived product Natustat™ (Duffy et al., 2005), several natural organic compounds (Grabensteiner et al., 2007) and other chemotherapeutics (tinidazole, metronidazole, ornidazole, doloxanide furoate, albendazole, mebendazole, paromomycin sulfate, 5,7-diiodo-8-hydroxy-quinoline, and carbadox) which are active against other protozoan parasites (Hu and McDougald, 2004).

A number of herbal products with claimed antibacterial and/or antiprotozoan activity have been marketed in recent years for addition to poultry feed or drinking water. However, most of these compounds have not been tested for antihistomonal activity. Therefore, the *in vitro* effect of some herbal products against *H. meleagridis* was examined in the present study.

An *in vitro* experiment is a cost-effective method to conveniently screen products for possible antihistomonal activity. An observed *in vitro* effect, however, may be caused by an antibacterial effect rather than a direct effect on the parasite since *H. meleagridis* growth is dependent on the presence of cecal bacteria in the dixenic culture (McDougald, 2005). Another shortcoming of an *in vitro* experiment is that the efficacy of compounds may be missed in case they only become active in the host, e.g., after metabolization (McDougald, 2005). Therefore, an observed *in vitro* effect should always be confirmed in an *in vivo* experiment.

Materials and Methods

Products. Two drugs with known activity against histomonosis were used as control products during the *in vitro* experiments: dimetridazole (1,2-dimethyl-5-nitroimidazole; Sigma, Zwijndrecht, The Netherlands) and Histostat-50™ (Alpharma Inc., Fort Lee, USA) consisting of 50% nitarsone (4-nitrophenylarsonic acid) with calcium carbonate and rice hull as carrier material.

Three herbal products were examined. Aromabiotic™ (Vitamix, Belgium) is a feed additive (advised dosage 0.3%) derived from tropical plants. It consists of the medium chain fatty acids capronic acid, caprylic acid and capric acid. Enteroguard™ (Orffa, Giessen, The Netherlands) is a feed additive (dosage 0.5–2%) consisting of lyophilized garlic and cinnamon infusion with active compounds allicin and cinnemaldehyde and other thiosulfonates. Protophyt™(Phytosynthèse, Riom, France) is a product based on oils extracted from leaves of cinnamon (8–12%), garlic (3–5%), rosemary (2–3%) and lemon (1–2%). Both the feed additive form of this product, Protophyt SP™ (dosage 0.3%), as well as the drinking water form, Protophyt B™ (dosage 0.3–1%), were examined.

H. meleagridis **isolate and culture**. The *H. meleagridis* strain Turkey/Deventer/NL/AL327-type I/03 (Van der Heijden et al., 2006) was isolated from a turkey presenting typical liver and caecal lesions (Van der Heijden et al., 2005). The isolate, stored at -175 °C in liquid nitrogen in 1 ml aliquots, was resuscitated in 12.5 ml of Dwyer medium (Dwyer, 1970) at 40 °C, and consisting of 85% Medium 199 with Hanks salts (Gibco-BRL), 10% heat-inactivated horse serum (Gibco-BRL), 5% chicken embryo homogenate of 8-day-old eggs and 12 mg of rice powder (Arrowhead Mills, Hereford, USA). After an incubation period of 3 days at 40 °C subculturing was performed by inoculating 1 ml of histomonad culture into five 70 ml tissue culture flasks (Becton Dickinson, France) containing 12.5 ml of modified Dwyer's medium prewarmed at 40 °C and consisting of 90% Medium 199, 10% horse serum and 100 mg (0.8%) of rice powder (Van der Heijden and Landman, 2005). After 24 h of incubation the contents of the five tissue culture flasks were pooled. The concentration of histomonads was determined using a Bürker-Türk haematocytometer and phase contrast microscopy at 200x magnification (minimum required 0.5 x 10^6 histomonads/ml).

In vitro **experiments**. Three *in vitro* experiments were performed. In the first experiment all products were tested at 400 µg/ml, or 0.24% (v/v) for Protophyt B™, in PBST (phosphate buffered saline with 0.2% Tween 80). In the second experiment, the products that had an inhibitory effect at the high concentration used in the first experiment, were tested serially diluted in PBST. In the third experiment, the products that were not effective in the first experiment, were tested serially diluted in PBS without Tween. In all experiments negative controls (PBST

or PBS) and positive controls (dimetridazole at 400 and 12.5 µg/ml) were included. All experiments were repeated.

Fresh stocks of the herbal products were prepared for each experiment by suspending 100 mg in 10 ml of PBST or PBS except for the liquid Protophyt B™ that was diluted 1:10. Suspensions were vortexed three times during 20 s and incubated for 1 h at room temperature. Then, the suspensions were vortexed again during 20 s. From these stock suspensions twofold serial dilutions were made in PBST or PBS using 1 ml volumes (concentrations in culture: 400, 200, 100, 50, 25, and 12.5 µg/ml). In each tissue culture flask, 7 ml of the pooled 24 h *H. meleagridis* culture was added to 5.5 ml of the freshly prepared modified Dwyer's medium prewarmed at 40 °C. Subsequently, 521 µl of the product suspension was added and mixed by gentle swirling. Cultures were incubated at 40 °C and counted at 8, 24, 32, 48, 54, and 72 h (during the first experiment also at 4, 20, 28, and 44 h).

Statistical analysis. The log-transformed duplicate parasite counts of cultures with product additions were compared to the relevant negative control (PBS or PBST) using Student's t-test with a 95% confidence interval.

Results

Experiment I. The histomonad counts for Enteroguard™, Aromabiotic™, Protophyt SP™, and the negative control PBST remained high throughout the experiment (Figure 1). Almost identical results were obtained when the experiment was repeated. For Protophyt B™, dimetridazole, and Histostat-50™ the number of parasites declined gradually with time. For Protophyt B™ the number of histomonads was already significantly lower than for PBST at 4 h of incubation and dropped below the detection limit ($10^{3.7}$ histomonads/ml) after 20 h, while for dimetridazole and Histostat-50™ the parasite counts for the first time were significantly lower than for PBST after 24 and 28 h of incubation, respectively.

Experiment II. No histomonads were detected throughout the experiment when 0.24% Protophyt B™ was added to the culture medium (Figure 2). At a concentration of 0.12% the number of histomonads decreased during the experiment and dropped below the detection limit after 54 h. Some inhibition of *H. meleagridis*' growth was also observed for 0.06% Protophyt B™. At repetition, the inhibitory effect of 0.24% Protophyt B™ was confirmed. However, at lower concentrations the numbers of histomonads did not decline over time. On average, significantly lower parasite counts were only found for 0.24% Protophyt B™ compared to PBST.

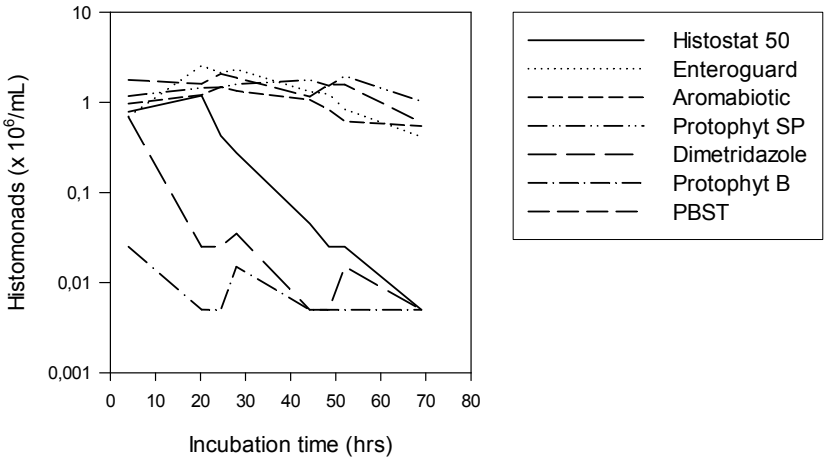


Figure 1. Histomonads counts of cultures with different products added at a concentration of 400 µg/ml (or 0.24% for Protophyt B™) in Phosphate Buffered Saline with Tween 80 (PBST).

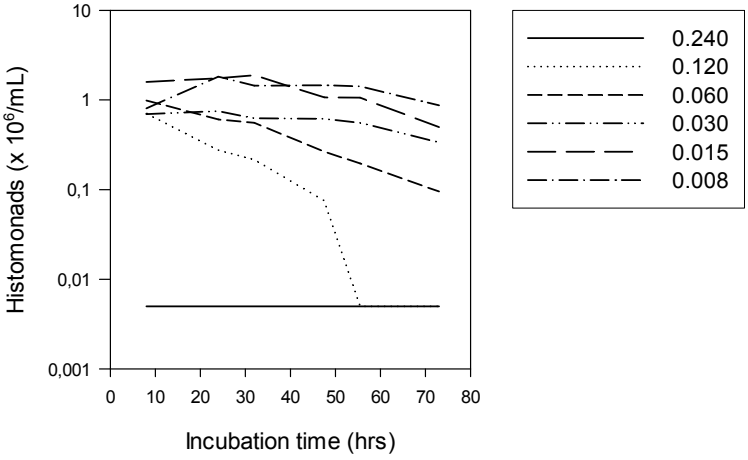


Figure 2. Histomonads counts of cultures with serial dilutions of Protophyt B™ (%) in PBST.

Complete elimination of histomonads from culture was observed after 32 h when dimetridazole was added to the culture medium at concentrations ranging from 50 to 400 µg/ml (Figure 3). At a concentration of 12.5 µg/ml dimetridazole induced a temporary decrease in histomonad counts until 32 h of incubation. Thereafter, the number of parasites increased again. Upon repetition, a similar effect was found, though now this concentration of 12.5 µg/ml dimetridazole did not

fully inhibit growth of histomonads at 32 h. Moreover, a temporary decrease in histomonads was also observed at a concentration of 25 µg/ml dimetridazole. On average, compared to PBST, significantly lower parasite counts for dimetridazole concentrations between 400 and 50 µg/ml were found from 24 h of incubation onwards.

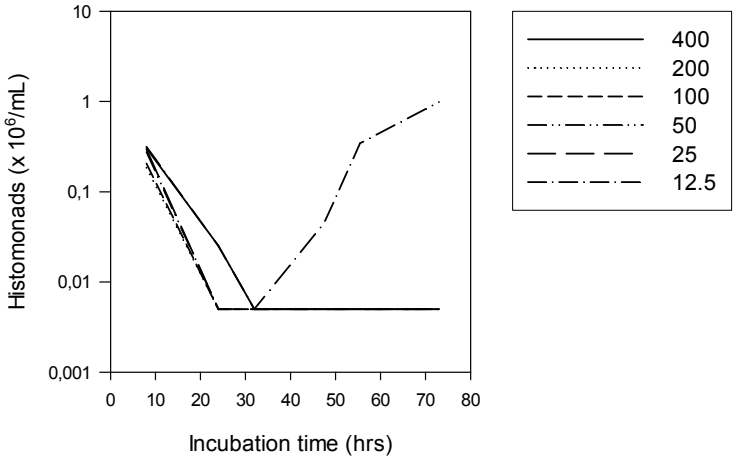


Figure 3. Histomonads counts of cultures with serial dilutions of dimetridazole (µg/ml) in PBST.

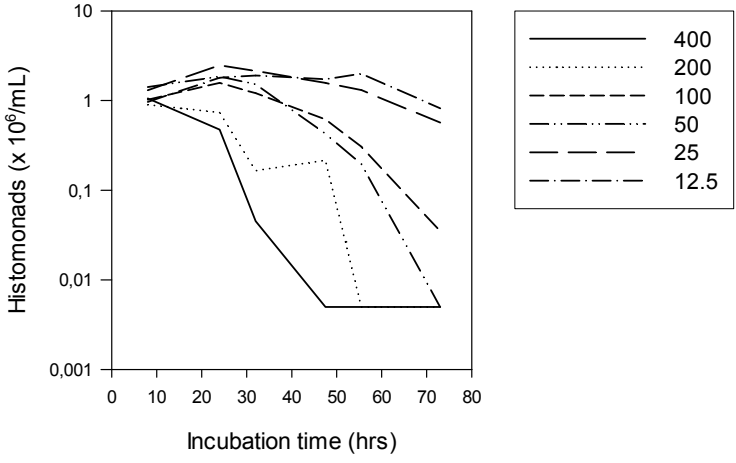


Figure 4. Histomonads counts of cultures with serial dilutions of Histostat-50™ (µg/ml) in PBST.

Histostat-50™ yielded significantly lower histomonad counts compared to PBST from 32 h of incubation onwards at concentrations of 400 and 200 µg/ml. A concentration of 50 µg/ml Histostat-50™ (Figure 4) also showed complete elimination of parasites, while this was not the case at a concentration of 100 µg/ml. Nevertheless, at repetition opposite results were obtained. Histostat-50™ concentrations of 25 or 12.5 µg/ml did not significantly affect parasite growth.

Experiment III. Three products (Aromabiotic™, Protophyt SP™, and Enteroguard™), that showed no inhibitory effect on histomonad growth when suspended in PBST (experiment I), were also tested after suspension and serial dilution in PBS (i.e., without Tween 80).

The numbers of histomonads were comparable throughout the experiment for both Aromabiotic™ and Protophyt SP™ at all concentrations tested and did not significantly differ from the counts of the negative control PBS.

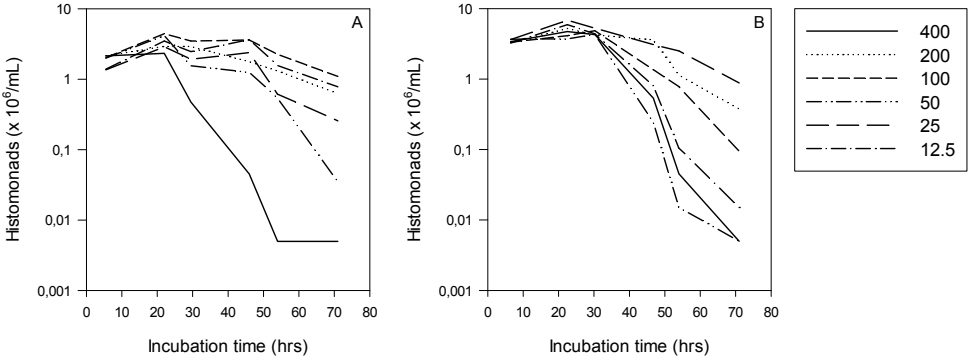


Figure 5. Histomonads counts of cultures with serial dilutions of Enteroguard™ (µg/ml) in PBS. A. First test; B. Repetition.

In the first test (Figure 5A) with Enteroguard™ at the highest concentration (400 µg/ml) already after 32 h lower numbers of histomonads were found. Regarding the other concentrations tested hardly or only a small (50 µg/ml) decrease in the numbers of parasites was observed toward the end of the first test. In the second test (Figure 5B) again the highest concentration (400 µg/ml) of Enteroguard™ resulted in a clear decrease of the number of histomonads, but after 48 h. Other concentrations resulted in lower counts. The timing and degree of count decrease was not well correlated with the concentration. On average, only the 400 and 200 µg/ml after 72 h of incubation had a significantly lower parasite count in comparison with PBS.

The control groups (dimetridazole in two concentrations as the positive control, or PBS as a negative control in fourfold) that were included in this test showed similar patterns as in previous experiments.

Discussion

During the last decade only a few studies have been published in which the activity of products against *H. meleagridis* was examined. Nitroimidazoles (Callait et al., 2002; Hu and McDougald, 2004) and furazolidone (Callait et al., 2002) were found to be effective chemotherapeutics as already reported in the early days of histomonas research. However, the withdrawal of these products (Byrne, 2001), because of toxic and possible carcinogenic properties, prompted the quest for alternative products with antihistomonal activity. Volatile oils extracted from leafs of *C. aromaticum*, *C. limon* and *A. sativum* were found to have an antihistomonal activity against *H. meleagridis in vitro* before (Zenner et al., 2003), and Grabensteiner et al. (2007) found *in vitro* susceptibility of *H. meleagridis* isolates to an essential oil mixture derived from thyme and rosemary, carvacrol, *Caissa* oil and *Quillaja saponaria* saponin.

In the present study, a number of feed additives and a drinking water additive were tested *in vitro*, with dimetridazole as a positive control with known antihistomonal activity. The effective dose of dimetridazole was found to be approximately 12.5 µg/ml. This is in agreement with results obtained in other studies (Callait et al., 2002; Hu and McDougald, 2004). At higher concentrations (400–50 µg/ml) the inhibition of growth was complete and irreversible throughout the test period, while partly and temporary inhibition of growth was observed at the lowest concentration tested (12.5 µg/ml). Probably a high concentration of dimetridazole is fatal to *H. meleagridis*, whereas at lower concentrations only a histomonostatic effect occurs. These results parallel other *in vitro* (Callait et al., 2002) and *in vivo* studies (McDougald, 2005).

The effective *in vitro* concentration of nitarsone (an arsenic compound) is not known. Histostat-50™ in the present study inhibited growth of parasites at concentrations of 50 µg/ml or higher. The exposure time needed to inhibit the growth of *H. meleagridis* was dose-dependent and varied between approximately 30 h (400 µg/ml) and more than 60 h (50 or 100 µg/ml), which was longer than the exposure time for dimetridazole. This delay may have been caused by the carrier material of Histostat-50™ partly consisting of rice hull. Such materials could stimulate growth of *H. meleagridis* during *in vitro* experiments, because rice powder is known to be an important constituent of the culture medium for *H. meleagridis* (Van der Heijden et al., 2005). Also other flours like corn, starch, rye, oat, barley, and buckwheat are suitable replacements for rice powder (McDougald, 2005).

In the present study Enteroguard™ (with the active compounds allicin from garlic and cinnemaldehyde from cinnamon) was effective against *H. meleagridis*. Tween 80 was found to mask the antihistomonal effect, since only inhibition of parasite growth was seen when the product was suspended and serially diluted in PBS without Tween 80. The masking effect was probably not directed against the active compounds since Zenner et al. (2003) found an *in vitro* antihistomonal activity in oils derived from leafs of garlic and cinnamon, and had also used Tween 80 as a detergent in their solvent.

The correlation between Enteroguard™ concentration and the antihistomonal effect was relatively poor and also the reproducibility antihistomonal effect was worse than that of the other products. A possible explanation could be the composition of the product. Non-effective components could serve as a nutrient for histomonads and subsequently mask the action of the antihistomonal compounds. This could lead to the irregular results observed in the present study.

The results of both Protophyt SP™ (feed additive) and Protophyt B™ (drinking water additive) were completely opposite. Protophyt B™, when tested at the highest concentration, showed a significant effect against *H. meleagridis*, while no antihistomonal effect was seen at all with Protophyt SP™. Both products are based on the same ingredients (volatile oils from garlic, cinnamon, rosemary and lemon). The difference might have been caused by differences in concentrations of the active compounds in the culture medium, e.g., due to a better dispersion of the volatile oils in the liquid product facilitated by the addition of emulsifiers.

Aromabiotic™ is based on medium chain fatty acids like capronic acid. The mode of action against bacteria is based on the assumption that these fatty acids incorporate in the cell wall structure and cause leakage of the cells. In addition, this product causes an intracellular rise in pH caused by dissociation. It is unknown whether the same mechanisms would apply to eukaryotic cells like histomonads. Nevertheless, in the present study no antihistomonal effect of Aromabiotic™ was found.

Although *in vitro* testing is a practical method to screen products or compounds for a possible antihistomonal effect, it has some drawbacks. The results are not always very reproducible; variation was observed in the time needed for inhibition of parasite growth, as well as in the minimal inhibitory concentrations. However, in a screening test it is more important that candidate products consistently inhibit histomonal growth rather than yielding exact *in vitro* inhibitory concentrations and incubation times. Further, the nature and composition of the solvent used in an *in vitro* study may also influence the results. Most feed additives contain carrier materials (e.g., rice hull) that cannot be dissolved and which may stimulate histomonal growth, while the use of active oily compounds requires the addition of detergents for their dissolvation.

In vitro testing of antihistomonal activity may also result in false positive results if the products are bactericidal to the cecal bacteria present in the *H. meleagridis*

culture (McDougald, 2005). It is generally accepted that *H. meleagridis* will only grow in dixenic culture with cecal bacteria or in axenic culture with killed bacteria added to the medium (Lesser, 1960). Indeed, using an antibiotic (streptomycin) complete inhibition of the cecal bacterial growth in the medium was achieved (data not shown). This also resulted in complete inhibition of growth of the *Histomonas* strain used in the present study. Remarkably, Callait et al. (2002) reported successful culture of *H. meleagridis* up to 48 h after specific destruction of the bacterial flora, concluding that the inhibition of histomonads growth in their study was directly caused by the product tested. In the present study not a single product caused a visually less turbid culture, suggesting that the products did not inhibit bacterial growth. However, as the intestinal flora is complex and consists of different species it cannot be excluded that a product may specifically eliminate one (or several) bacterial species that are essential for the growth of *H. meleagridis*. This is another reason why it is very important to confirm the *in vitro* efficacy of products in an *in vivo* study. Therefore, the observed antihistomonal *in vitro* effects of Protophyt B™ and Enteroguard™ should be confirmed in an animal experiment before final conclusions about their antihistomonal efficacy can be drawn.

Acknowledgements

This work was financially supported by nine Dutch turkey feed mills: Agrifirm B.V., Boerenbond Deurne, Boerenbond Ysselsteyn Mengvoeders B.V., Brameco-Zon Voeders, Cehave Landbouwbelang Voeders B.V., CoöperatieAgruniek u.a., De Heus Voeders B.V., Isidorus Mengvoeders B.V., and Rijnvallei Diervoeder B.V.

References

Byrne, D., 2001, Commission regulation EC no. 2205/2001, amending council directive 70/524/EEC concerning additives in feeding stuffs as regards withdrawal of the authorization of certain additives. Official Journal of the European Community L297, 3-4.

Callait, M.P., Granier, C., Chauve, C., Zenner, L., 2002, *In vitro* activity of therapeutic drugs against *Histomonas meleagridis* (Smith, 1895). Poultry. Science 81, 1122-1127.

Duffy, C.F., Sims, M.D., Power, R.F., 2005, Evaluation of dietary Natustat™ for control of *Histomonas meleagridis* in male turkeys on infected litter. Avian Diseases 49, 423-425.

Dwyer, D.M., 1970, An improved medium for cultivating *Histomonas meleagridis*. Journal of Parasitology 56, 191-192.

Grabensteiner, E., Arshad, N., Hess, M., 2007, Differences in the *in vitro* susceptibility of mono-eukaryotic cultures of *Histomonas meleagridis*, *Tetratrichomonas gallinarum* and *Blastocystis* sp. to natural organic compounds. Parasitology Research 1, 193-199.

Graybill, H.W., Smith, T., 1920, Production of fatal blackhead in turkeys by feeding embryonated eggs of *Heterakis papillosa*. Journal of Experimental Medicine 31, 647-655.

Hegngi, F.N., Doerr, J., Cummings, T.S., Schwartz, R.D., Saunders, G., Zajaz, A., Larsen, C.T., Pierson, F.W., 1999, The effectiveness of benzimidazole derivatives for the treatment and prevention of histomoniasis (blackhead) in turkeys. Veterinary Parasitology 81, 9-37.

Hu, J., McDougald, L.R., 2004, The efficacy of some drugs with known antiprotozoal activity against *Histomonas meleagridis* in chickens. Veterinary Parasitology 21, 233-238.

Lesser, E., 1960, Cultivation of *Histomonas meleagridis* in modified tissue culture media. Journal of Parasitology 46, 686.

McDougald, L.R., 1997, Protozoa: other protozoan diseases of the intestinal tract. In: Calneck, B., Barnes, H.J., Beard, C.W., McDougald, L.R., Saif, Y.M. (Eds.), Diseases of Poultry. 10th ed. Iowa State University Press, Ames, pp. 890-899.

McDougald, L.R., 2005, Blackhead disease (histomoniasis) in poultry: a critical review. Avian Diseases 49, 462-476.

Van der Heijden, H.M.J.F., Landman, W.J.M., 2007, Improved culture of *Histomonas meleagridis* in a modification of Dwyer medium. Avian Diseases 51, 986-988.

Van der Heijden, H.M.J.F., McDougald, L.R., Landman, W.J.M., 2005, High yield of parasites and prolonged *in vitro* culture of *Histomonas meleagridis*. Avian Pathology 34, 505-508.

Van der Heijden, H.M.J.F., Landman, W.J.M., Greve, S., Peek, R., 2006, Genotyping of *Histomonas meleagridis* isolates based on internal transcribed spacer-1 sequences. Avian Pathology 35, 330-334.

Zenner, L., Callait, M.P., Granier, C., Chauve, C., 2003, *In vitro* effect of essential oils from *Cinnamomum aromaticum*, *Citrus limon* and *Allium sativum* on two intestinal flagellates of poultry, *Tetratrichomonas gallinarum* and *Histomonas meleagridis*. Parasite 10, 153-157.

Chapter 3.2

In vivo effect of herbal products against *Histomonas meleagridis* in turkeys

H.M.J.F. van der Heijden and W.J.M. Landman

Animal Health Service Ltd, P.O. Box 9, 7400 AA Deventer, the Netherlands

Published: Avian Pathology, 37 (2008) 45-50

Abstract

Histomonosis is a serious disease in poultry. All chemotherapeutics with known efficacy against its causative agent, *Histomonas meleagridis*, have been banned from use as prophylactic or therapeutic use in production animals. In a search for possible alternatives, the *in vivo* effects of the herbal products Enteroguard™ and Protophyt™ were examined. Two-week-old turkeys allocated into 13 groups of 18 birds were either sham inoculated (negative control group) or were inoculated with 100, 3162 or 200 000 histomonads per bird. Control groups (no feed additives, dimetridazole, or Histostat-50™) were included in the study. No morbidity or mortality was observed in the negative control group or in the groups inoculated with 100 histomonads per bird. Mortality was 100% in the groups inoculated with 200 000 histomonads per bird and either untreated (positive control group) or receiving Protophyt SP™, Protophyt™ SP and Protophyt™ B, Enteroguard™, or Histostat-50™. Mortality was 17% in the dimetridazole-treated group. In the groups inoculated with 3162 histomonads per bird, mortality was 100% for the positive control group and the group receiving Enteroguard™, and was 94% in the group receiving Protophyt™ SP. In the present study, Enteroguard™ or Protophyt™ was not found to be effective against histomonosis.

Introduction

Histomonosis (infectious enterohepatitis, blackhead disease) is a disease of galliforms and other species of birds, mainly affecting the liver and caecae. The severity of the disease varies over the different species. In turkey flocks, for example, mortality can be very high, whereas in chickens the symptoms are generally less severe (McDougald, 1997). The causative agent of histomonosis, *Histomonas meleagridis*, is transmitted between flocks through the vector *Heterakis gallinarum* (Graybill and Smith, 1920). Within a flock, direct lateral transmission of *H. meleagridis* occurs between birds (Hu and McDougald, 2003).

Effective chemotherapeutics such as nifursol and nitroimidazoles are no longer allowed in both the United States and the European Union (McDougald, 2005). In addition, the European Union also banned arsenical compounds (Byrne, 2001). Therefore, there is an urgent need for alternative antihistomonal products (Hu and McDougald, 2004). In the field, herbal products are used to prevent outbreaks of histomonosis (Hafez and Hauck, 2006). However, there are only a few scientific *in vivo* studies backing up the efficacy of such products against *H. meleagridis*. Duffy et al. (2005) observed a positive effect of Natustat™, a yeast-derived mannonoligosaccharide combined with organic mineral nutrients and plant extracts on caecal and liver lesions scores but not on mortality. In a recent study (Hafez and Hauck, 2006), Protophyt™ SP (feed additive) and Protophyt™ B (drinking water additive) were found to reduce mortality following experimental inoculation with *H. meleagridis* from 50% (untreated group) to 20% (additives), with no mortality in the control group. In the present study the antihistomonal effects of Enteroguard™ and Protophyt™ are examined on turkey poults that were experimentally inoculated with three different inoculation doses of histomonads. Enteroguard™ is a product based on garlic and cinnamon, with allicin and cinnemaldehyde as possibly active compounds. Protophyt™ is based on volatile oils extracted from garlic, cinnamon, rosemary and lemon.

Materials and Methods

Medicated feed and drinking water. Non-medicated turkey starter feed (no. 94040; Arkervaart-Twente, Nijkerk, the Netherlands) and nonmedicated turkey feed (no. 94041; Arkervaart-Twente) were mixed with the different antihistomonal products: either 200 ppm dimetridazole (1,2 dimethyl-5-nitroimidazole, D4025; Sigma, Zwijndrecht, the Netherlands) or 375 ppm Histostat-50™ (no. 560101; Alpharma Inc., Fort Lee, USA), or 3000 ppm Protophyt SP™ (Phytosynthèse, Riom, France) or 500 ppm Enteroguard™ (Orffa, Giessen, the Netherlands). To ensure an adequate distribution of the product in the feed, the following feed mixing procedure was used. A maximum quantity of 25 kg feed was mixed per run. After

accurate weighing of the product it was transferred to a plastic bag. Approximately 500 g feed was added and thoroughly mixed. Subsequently, the contents of the bag were transferred to a larger plastic bag that contained approximately 2.5 kg feed, followed by thorough mixing. During the next mixing step another 2.5 kg feed was added and mixed again. Thereafter, the contents of the bag were mixed with the remainder of the 25 kg feed for approximately 15 min using a blender (no. 19091; Naturamix, Haarlem, the Netherlands). The medicated feed was collected in a labelled paper bag. All of the medicated feed needed for the whole experimental period, except for the dimetridazole feed, was produced within 2 days and was stored at room temperature until use. The dimetridazole feed was freshly mixed every week and stored at 4 °C in the dark, as recommended by the manufacturer. The blender was thoroughly cleaned between products using a brush, followed by operation of the blender with 5 kg non-medicated feed that was subsequently discarded.

Drinking water with a herbal additive (Protophyt™ B) was freshly prepared every day by adding 2 ml Protophyt B™ per litre of water.

Assessment of feed mixing procedure. Samples were taken from the feeds with dimetridazole and Histostat-50™ and were analysed for dimetridazole and arsenic content to assess the mixing procedure.

Dimetridazole was quantified by high-performance liquid chromatography after extraction with acetonitrile/methanol. Briefly, 5 g feed was moistened with 15.0 ml water. After 5 min, 35.0 ml of a mixture (1:1) of acetonitrile (Fisher Scientific, Loughborough, UK) and methanol (Biosolve, Valkenswaard, the Netherlands) was added. The suspension was shaken for 30 min at room temperature. The extract was filtered through a paper filter (Schleicher, Dassel, Germany) and eluted over 4 g neutral alumoniumoxide (Fisher Scientific) packed in a glass column (25 ml). The first 2 ml eluted filtrate was not used. The extract was diluted 10 times with mobile phase. The mobile phase consisted of 170 ml sodium-acetate buffer (0.01 mol/l pH 6.0) with 30 ml acetonitrile. The high-performance liquid chromatography column was a 3.0 mm C18 Chromspher packed with 40 µm reversed phase material (Varian, Middelburg, the Netherlands). Dimetridazole was quantified against standard material (Rhône-Poulenc, Amstelveen, the Netherlands) at 320 nm. All handling was done under yellow light to protect degradation of dimetridazole. The detection limit was 0.8 mg/kg. The recovery of the method is between 90% and 95% in the range of 2 to 500 mg/kg.

Arsene was determined by Inductively Coupled Plasma (ICP). Briefly, 1 g feed to which 6 ml of 70% HNO3 (Baker Chemicals, Deventer, the Netherlands) had been added was destructed in a magnetron digestion unit (Milestone Inc., Shelton, USA) until a clear destruate remained. After complete solubilization, 50 ml water was added. Arsene was measured with an ICP analyser (Optima 3300 DV; Perkin Elmer, Waltham, USA).

Experimental set-up. In two separate rooms (6.85 m x 4.48 m), room A and room B, 13 stainless steel wire pens (1.3 m x 1 m) were constructed. On the left side of both rooms four pens (groups 1 to 4, and groups 8 to 11, respectively) were positioned at a distance of 40 cm, while on the right side of the rooms three pens (groups 5 to 7) or two pens (groups 12 and 13) were placed, also at a distance of 40 cm. The side walls of all pens were covered with plastic to minimize the risk of cross-infections between pens. Approximately 8 cm wood shavings was used as bedding litter. The experimental rooms were only accessible for qualified personnel, and involved changing of footwear and overall clothing.

One-day-old BUT Big 6 poults (120 females and 120 males) were kept separately in two pens for 1 week, with non-medicated drinking water and feed (turkey starter) *ad libitum*. The lighting program was 2 h of darkness and 22 h of light.

After 1 week all birds were tagged (Swifttack), individually weighed and divided into six weight classes per sex. All birds of each weight class were then randomly distributed over the 13 pens. Each group consisted of nine female and nine male poults. Until the termination of the experiment at the age of 6 weeks, light was provided for 16 h per day.

The experimental groups 1 to 7 (Table 1) in room A were given a ‘‘standard’’ inoculation dose of 200 000 histomonads per bird, whereas the birds in room B received either a low inoculation dose of 3162 histomonads per bird (groups 8, 10, and 12) or a very low inoculation dose of 100 histomonads per bird (groups 9, 11, and 13). The inoculations were done during two consecutive days in room A, and room B, respectively. The negative control group (group 2, no treatment and no inoculation) was directly positioned next to the positive control group (group 1, inoculated but not treated). During handling of the animals throughout the experiment (feed supply, removal of dead birds from the pens, etc.) the negative control group was always handled last in order to detect possible cross-infection.

Table 1. Experimental groups and inoculation dose

Experimental room	Group	Feed additive	Dose (histomonads/bird)
A	1	No (positive control)	200 000
	2	No (negative control)	0
	3	Dimetridazole	200 000
	4	Histostat-50™	200 000
	5	Protophyt SP™	200 000
	6	Enteroguard™	200 000
	7	Protophyt SP/B™	200 000
B	8	No (positive control)	3162
	9	No (positive control)	100
	10	Protophyt SP™	3162
	11	Protophyt SP™	100
	12	Enteroguard™	3162
	13	Enteroguard™	100

At the age of 7 days (i.e. at transfer of the animals to the experimental groups), the starter feed with additives was supplied *ad libitum*. However, non-medicated feed and drinking water was continued in group 7 until 2 days before inoculation, when starter feed with Protophyt SP™ and drinking water with Protophyt B™ was supplied to the birds.

At the time of inoculation at 2 weeks of age, all groups changed from turkey starter feed to turkey feed with the appropriate feed additive for the remainder of the experimental period (until 6 weeks of age).

After inoculation, the groups were inspected three times a day and dead birds removed from the pens. When birds were very sick and did not eat for two consecutive inspections, these were euthanized by injection with T61.

***H. meleagridis* strain**. Strain /Deventer/NL/AL327-type I/03 (Van der Heijden et al., 2006), a Dutch field strain that was successfully propagated in culture (Van der Heijden et al., 2005), was used for inoculation. On two consecutive days, the isolate was resuscitated, cultured in Dwyer's medium (Dwyer, 1970), and subcultured once in modified Dwyer's medium (Van der Heijden et al., 2007) consisting of Medium 199 with Hanks salts (Gibco-BRL, Grand Island, USA) with 10% heat-inactivated horse serum (Gibco-BRL) and 0.096% w/v white rice powder (Arrowhead Mills Inc., Hereford, USA). After pooling of the contents of four tissue culture flasks, a 1:5 pre-dilution was made in pre-warmed Medium 199 with Hanks salts and counted using a Bürker-Türk haemocytometer and phase contrast microscopy at 200x magnification. Subsequently, the suspensions were further diluted in M199 medium to obtain a concentration of 200 000 histomonads/ml and were counted before and after the inoculation on both inoculation days. On the second inoculation day, the 200 000 histomonads/ml suspension was further diluted in order to obtain the desired inoculation doses (Table 1). The inoculation doses were aliquoted for each experimental group by pipetting 25 ml into pre-warmed 50 ml tubes that were kept warm until use.

Inoculation. The turkeys were essentially inoculated by the method of Chappel (1975). The birds were inoculated intracloacally with 1 ml histomonad suspension using a 10 ml syringe (Pharma-Plast) with a blunt tip. After inoculation a finger was placed over the cloaca for 30 sec to prevent voiding of the inoculum. Subsequently the birds were suspended in inverted position for a maximum of 5 min but at least for the time that the cloaca was observed pulsating (usually for approximately 1 min after stimulation). In only three out of 234 cases was voiding of the inoculum seen (birds in groups 3, 7 and 12), after which these turkeys received a new inoculum. No birds were seen discharging after return to their pens. The negative control group was inoculated with 1 ml M199 medium instead of histomonads.

Postmortem examination. Thorough postmortem examination was performed on all experimental birds either during the experiment (in case of mortality or severe disease) or at the end of the study at the age of 6 weeks. Birds that died during the experiment were kept at 4 °C before being subjected to postmortem examination within 1 or 2 days. Six weeks after inoculation the remaining turkeys were transported in boxes to the postmortem room and euthanized using carbon dioxide. At necropsy, liver and caecal lesions were scored on a scale ranging from 0 (no lesions) to 4 (McDougald and Hu, 2001).

Statistical analysis. The time of survival in the groups with different treatments was analysed using one-way analysis of variance. Group means were adjusted using Bonferroni's method. The residuals were assessed for being normally distributed. The liver and caecum lesion scores between groups were analysed using the non-parametric Kruskal-Wallis comparison test. Statistical analysis was performed using Statistix 8 (Analytical Software, Tallahassee, USA).

Results

Assessment of the feed mixing procedure. The average dimitridazole level of the feed mixed with dimetridazole was 182 ppm (desired dose 200 ppm), while the average arsenic level of the feed mixed with Histostat-50™ was 68 ppm (desired dose 63 ppm), which corresponds to 405 ppm Histostat-50™ (desired dose 375 ppm).

***H. meleagridis* inoculation**. Not a single turkey died in the negative control group (no treatment, sham inoculated) and at postmortem the livers and caecae of all birds did not show abnormalities (all were scored 0). In the experimental groups inoculated with the standard dose of 200 000 histomonads per animal (Figure 1), mortality was high in most of the groups. The first turkeys died from histomonosis around day 10 postinoculation (p.i.). In the positive control group (no feed additive) and in the groups treated with either Protophyt SP™ or Enteroguard™ at day 13 p.i. approximately 50% of the animals had died, while 50% mortality was (slightly) delayed by 1 or 2 days in the experimental group with combined therapy with Protophyt SP™ and Protophyt B™ and in the group treated with Histostat-50™. At days 16 to 18 p.i. almost all animals had died in the positive control group and in the groups treated with either Protophyt™ or Enteroguard™, while 100% mortality was delayed for 3 days in the group medicated with Histostat-50™. In the experimental group that received dimetridazole, the first two turkeys died at 17 days p.i. Nevertheless, mortality in this group was limited to 17% at 30 days p.i. when the experiment was terminated. Surprisingly, 13 of the 15 surviving animals in this group presented typical lesions in the caecae and liver at necropsy.

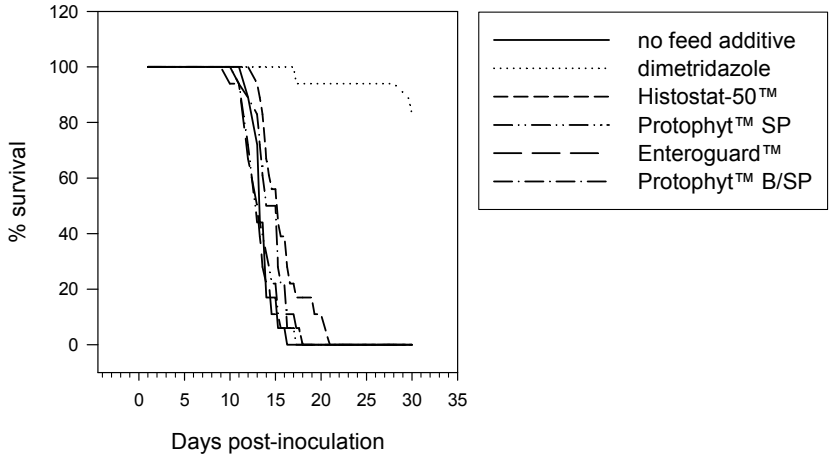


Figure 1. Survival in experimental groups following inoculation with 200 000 histomonads per turkey.

The different treatments of the groups in which the turkeys were inoculated with 200 000 histomonads per bird had a significant effect (one-way analysis of variance, $P<0.05$) on the time of survival -and also when the dimetridazole group was left out of the analyses (Table 2). Only in the groups receiving dimetridazole or Histostat-50™ did birds die significantly later than in the positive control group (Bonferroni's pair-wise comparisons test, $P<0.05$). Among these groups the effect of treatment was significant for the liver lesion scores (Kruskal-Wallis, $P<0.05$) but not for caecal lesion scores ($P>0.05$). Pair-wise comparisons revealed no differences between the groups in liver lesion scores.

The results in the three groups inoculated with the low dose of 3162 histomonads per animal (Figure 2) were very similar. The first turkeys died in the positive control group (no feed additive) at 7 days p.i., even prior to the positive control group inoculated with 200 000 histomonads per animal. In the other two groups treated with Protophyt SP™ or Enteroguard™, mortality was first seen at 9 or 12 days p.i., respectively. In all three groups 50% mortality occurred at 15 days p.i., which was 2 days later than the groups inoculated with 200 000 histomonads per animal. Finally, maximum mortality occurred at 19 days p.i., also slightly delayed compared with the groups inoculated with 200 000 histomonads per bird. The single turkey in the Protophyt SP™ group that survived throughout the experiment had no lesions in the liver or caecae upon necropsy.

Table 2. Comparison of mortality day and ceca and liver lesions scores of turkeys that died following inoculation with *H. meleagridis*.

Dose (Hm/bird)	Feed additive	n[a]	Mortality day		Ceca lesions		Liver lesions	
			Mean	SD[b]	Mean	SD	Mean	SD
0	No additive	0	-	-	-	-	-	-
100	No additive	0	-	-	-	-	-	-
	Protophyt SP™	0	-	-	-	-	-	-
	Enteroguard™	0	-	-	-	-	-	-
3162	No additive	18	15.1	2.68	3.9	0.24	3.1	0.90
	Protophyt SP™	17	15.1	2.30	3.9	0.24	3.0	0.79
	Enteroguard™	18	15.5	1.69	4.0	0.00	3.3	0.49
200 000	Dimetridazole	3	25.7^{A}	7.25	3.3	0.58	3.3	0.58
	Histostat-50™	18	16.2^{B}	3.25	3.9	0.32	3.0	0.59
	Protophyt SP/B™	18	14.5^{BC}	1.74	3.9	0.32	3.4	0.51
	No additive	18	13.7^{C}	1.15	3.9	0.24	3.3	0.46
	Protophyt SP™	18	13.3^{C}	1.45	3.9	0.24	3.6	0.50
	Enteroguard™	18	13.5^{C}	1.82	4.0	0.00	3.4	0.51

Means with different superscripts are significantly (P<0.05) different among the groups with the same infective dose.
[a]Number of birds that died during the experiment.
[b]Standard deviation.

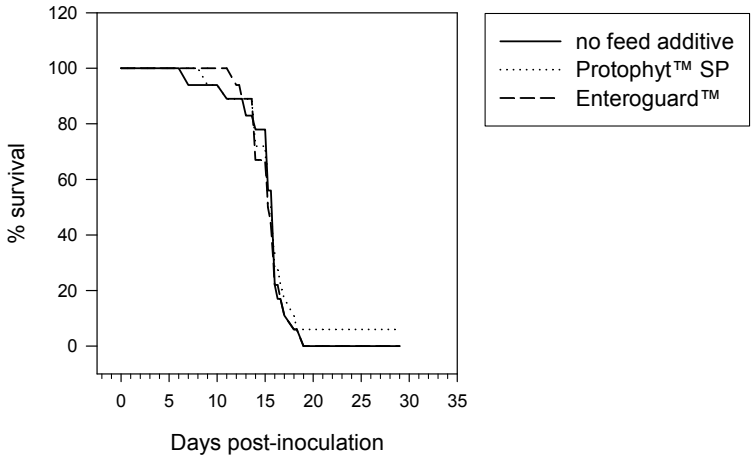


Figure 2. Survival in experimental groups following inoculation with 3162 histomonads per turkey.

No significant effect of treatment in the groups inoculated with 3162 histomonads per bird was found, either for number of days of survival (one-way

analysis of variance, P>0.05) or for caecal or liver lesion scores (Kruskal-Wallis, P>0.05).

In the three experimental groups inoculated with 100 histomonads per animal and either non-treated (positive control, no feed additive) or treated with Enteroguard™ or Protophyt SP™, no mortality was seen and the livers and caecae were normal (score 0) at postmortem.

No differences were seen in mortality of female or male turkeys after experimental inoculation with *H. meleagridis* (Figure 3). Additionally, there was no significant relation (regression analysis, P>0.05) between the weight of the poults at the start of the experiment and the number of days the bird survived inoculation, even if female and males results were analysed separately. In the groups inoculated with 100 histomonads per bird (i.e. no mortality), the overall consumption of feed with either no additive, Protophyt™ SP or Enteroguard was 32.5, 33.1, or 32.3 kg/group, respectively.

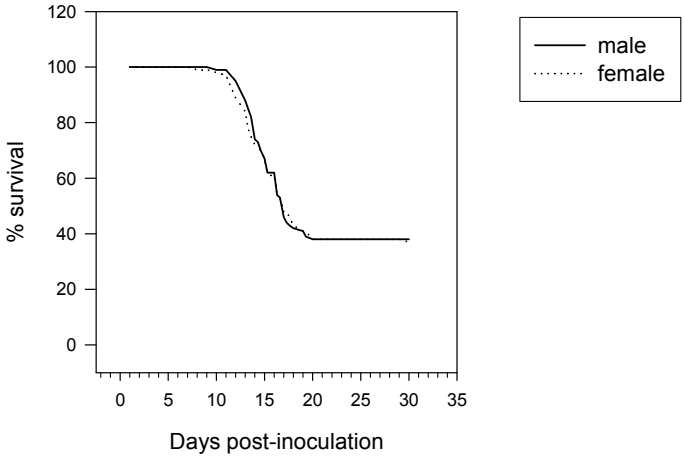


Figure 3. Comparison of survival of male and female turkeys among all groups after experimental inoculation with *H. meleagridis*.

Discussion

The ban of effective chemotherapeutics against *H. meleagridis* in commercial poultry (Byrne, 2001) propelled the search for alternative antihistomonal products. In the present study, two herbal products were tested for a possible anti-histomonal effect. However, both Protophyt™ and Enteroguard™ had no effect on mortality following inoculation of turkey poults with 200 000 histomonads per bird. In the positive control group, in the groups receiving either Enteroguard™ or Protophyt™

SP as a feed additive, and in the group of turkeys that received Protophyt™ SP as feed additive and Protophyt™ B in drinking water, mortality was 100%. The latter finding is in agreement with the results of a field study in turkeys (Chossat, 2002), but in contrast with a recent *in vivo* experiment (Hafez and Hauck, 2006). In the latter study turkeys provided with Protophyt™ SP in the feed 6 days before inoculation and Protophyt™ B in the drinking water 3 days before inoculation showed 20% mortality, instead of 50% mortality in the positive control group, after experimental inoculation with 147 500 histomonads per bird. This difference was only just significant (chi-square, P=0.047). Moreover, their birds were housed in cages, making re-infections through direct lateral transfer as under practical conditions less likely. The only positive effect of the Protophyt™ SP and Protophyt™ B treatment in the present study was a slight delay in mortality of 1 or 2 days in comparison with the other groups. Here, both Protophyt™ SP and Protophyt™ B were supplied only 2 days before inoculation. Possibly more time is needed for the products to partly protect birds against histomonosis following an inoculation with *H. meleagridis*.

Surprisingly, the mortality in the group medicated with Histostat-50™ was 100%. Nitarsone (4-nitrophenylarsonic acid) is the active compound of this product, and has been reported to be highly effective for preventive use in feed, although it is probably histomonastatic since a relapse has been described after withdrawal of medication (McGuire and Morehouse, 1952). In addition, some mortality was seen in the experimental group medicated with dimetridazole, especially toward the end of the experiment at 4 weeks after inoculation. Moreover, a large proportion of the surviving turkeys showed severe lesions at necropsy and it is probable that more turkeys would have died had the experiment been continued. Since dimetridazole is considered highly effective (McDougald, 2005) this result was unexpected. It is unlikely that this was caused by a slightly lower dose of dimetridazole (182 ppm instead of 200 ppm) in the feed since the 50% protective dose of dimetridazole was found to be 38 ppm (Lucas, 1961), and with 100 ppm (McGuire et al., 1963) or 125 ppm (Lucas and Goose, 1965) close to 100% protection against histomonosis was obtained. Also, the recovery of the dimetridazole test was determined during the validation of the assay and found to be 87% in the range of 5 to 100 ppm. It is expected that for higher concentrations up to 200 ppm the recovery is approximately the same. Therefore, the actual dimetridazole concentration in the feed was probably close to the desired 200 ppm.

A dose-response relation between the size of the infective dose and mortality was reported by Lund (1955). After inoculation with 10^5, 10^4, 10^3, 10^2, or 10 histomonads per turkey, mortality was 100%, 82%, 25%, 5%, and 5%, respectively. But the infective dose in itself probably also does not explain the results of the present experiment. Most inoculation studies used infective doses between 10^5 and 10^6 histomonads per animal (McDougald, 2005). In addition, in the present experiment a lower (3162) and a much lower (100) inoculation dose were

examined, although only in positive control groups and in groups of birds having either Protophyt SP™ or Enteroguard™ as a feed additive. In the groups inoculated with 3162 histomonads per bird, again all birds of the positive control group and the group treated with Enteroguard™ died. In the group of turkeys that was treated with Protophyt SP™ only a single bird survived the experiment. Thus, also at lower inoculation doses, the herbal products had no effect or hardly any effect on mortality. In all three groups inoculated with 100 histomonads per bird, however, not a single bird died during the experiment or showed lesions at necropsy.

The relative high virulence of the strain used might explain the fact that mortality was high, despite a relatively low inoculation dose. This might also be the cause of the considerable morbidity in the dimetridazole treated group towards the end of the experiment. This is speculative, however, since there have been no reports on the effect of chemotherapeutics on strains of different virulence, and it needs further investigation.

The inoculation procedure (Chappel, 1975) was probably very effective as none of the experimental poults (except for a few birds that were re-inoculated) showed discharge of the inoculum after returning to their pens. This may also have contributed to the higher mortality found compared with other *in vivo* studies using a different intracloacal inoculation method. On the other hand, infected birds are known to shed *H. meleagridis* as early as 2 days after inoculation (McDougald, 2005; Hess et al., 2006). Since the experimental turkeys were housed on litter direct lateral transmission of the parasite (Hu and McDougald, 2003), causing re-infections could have contributed to the higher mortality.

A possible explanation for the high mortality in the Histostat-50™ medicated group of birds may be that the administration of this feed additive started just 1 week before inoculation. This may have been too short for prophylactic treatment. Nitarsone, the active compound in Histostat-50™, is a prophylactic rather than a curative drug (Hu and McDougald, 2004).

Cross-contamination between groups, potentially masking an antihistomonal effect of a certain product, was highly unlikely. The negative control group (no medication, no inoculation) that was placed directly next to the positive control group with an inoculation dose of 200 000 histomonads per turkey remained free from clinical disease throughout the experiment and the birds showed no lesions at necropsy. The same was true in the experimental groups inoculated with 100 histomonads per bird as these were placed next to the groups inoculated with 3162 histomonads per bird and also remained free of disease, while almost every bird in the neighbouring pen died of histomonosis. No difference was found between female and male birds regarding the susceptibility to an experimental inoculation with *H. meleagridis*.

Acknowledgements

This study was funded by the commodity of the Board for Poultry and Eggs. The authors thank Orffa Additives (the Netherlands) and Biosynthèse (France) for providing Enteroguard™ and Protophyt™, respectively. They also thank Johan Welner and colleagues for their assistance in the biotechnological handling of the experimental birds.

References

Byrne, D., 2001, Commission regulation EC No. 2205/2001, amending council directive 70/524/EEC concerning additives in feeding stuffs as regards withdrawal of the authorization of certain additives. Official Journal of the European Communities L297, 3-4.

Chappel, L.R., 1975, Using the HmL1 strain of *Histomonas meleagridis* in evaluating chemotherapeutants in chicks. Avian Diseases 19, 679-682.

Chossat, L., 2002, L'Histomonose en production A.O.C. ''Dinde fermiere de Bresse'' essai de prevention par phytotherapie. Doctoral thesis, École Nationale Veterinaire D'Alfort Lyon.

Duffy, C.F., Sims, M.D., Power, R.F., 2005, Evaluation of dietary Natustat™ for control of *Histomonas meleagridis* in male turkeys on infected litter. Avian Diseases 49, 423-425.

Dwyer, D.M., 1970, An improved medium for cultivating *Histomonas meleagridis*. Journal of Parasitology 56, 191-192.

Graybill, H.W., Smith, T., 1920, Production of fatal blackhead in turkeys by feeding embryonated eggs of *Heterakis papillosa*. Journal of Experimental Medicine 31, 647-655.

Hafez, M.H., Hauck, R., 2006, Efficacy of an herbal product against *Histomonas meleagridis* after experimental infection of turkey poults. Archives of Animal Nutrition 60, 436-442.

Hess, M., Grabensteiner, E., Liebhart, D., 2006, Rapid transmission of the protozoan parasite *Histomonas meleagridis* in turkeys and specific pathogen free chickens following cloacal infection with a mono-eukaryotic culture. Avian Pathology 34, 280-285.

Hu, J., McDougald, L.R., 2003, Direct lateral transmission of *Histomonas meleagridis* in turkeys. Avian Diseases 47, 489-492.

Hu, J., McDougald, L.R., 2004, The efficacy of some drugs with known antiprotozoal activity against *Histomonas meleagridis* in chickens. Veterinary Parasitology 121, 233-238.

Lucas, J.M.S., 1961, 1,2-Dimethyl-5-nitroimidazole, 8595 R.P. Part I. Prophylactic activity against experimental histomoniasis in turkeys. Veterinary Record 73, 465-467.

Lucas, J.M.S., Goose, J., 1965, Tests for activity against *Histomonas meleagridis*: use of the infection in chickens and a comparison of the results obtained in chickens and turkeys. British Veterinary Journal 121, 65-75.

Lund, E., 1955, The progress of histomoniasis (Blackhead) in turkeys as related to the size of the infective dose. Poultry Science 34, 127-130.

McDougald, L.R., 1997, Protozoa: other protozoan diseases of the intestinal tract. In: Calneck, B., Barnes, H.J., Beard, C.W., McDougald, L.R., Saif, Y.M. (Eds.), Diseases of Poultry. 10th ed. Iowa State University Press, Ames, pp. 890-899.

McDougald, L.R., 2005, Blackhead disease (histomoniasis) in poultry: a critical review. Avian Diseases 49, 462-476.

McDougald, L.R., Hu, J., 2001, Blackhead disease (*Histomonas meleagridis*) aggravated in broiler chicks by concurrent infection with caecal coccidiosis (*Eimeria tenella*). Avian Diseases 45, 307-312.

McGuire, W.C., Morehouse, N.F., 1952, Chemotherapy studies of histomoniasis. Poultry Science 31, 603-609.

McGuire, W.C., Moeller, M.W., Morehouse, N.F., 1963, The effect of dimetridazole on growth and the prevention of histomonosis in poultry. Poultry Science 43, 864-871.

Van der Heijden, H.M.J.F., McDougald, L.R., Landman, W.J.M., 2005, High yield of parasites and prolonged *in vitro* culture of *Histomonas meleagridis*. Avian Pathology 34, 505-508.
Van der Heijden, H.M.J.F., Landman, W.J.M., Greve, S., Peek, R., 2006, Genotyping of *Histomonas meleagridis* isolates based on internal transcribed spacer-1 sequences. Avian Pathology 35, 330-334.
Van der Heijden, H.M.J.F., Landman, W.J.M., 2007, Improved culture of *Histomonas meleagridis* in a modification of Dwyer medium. Avian Diseases 51, 986-988.

Chapter 3.3

Assessment of the antihistomonal effect of paromomycin and tiamulin

H.M.J.F. van der Heijden and W.J.M. Landman

Animal Health Service Ltd, P.O. Box 9, 7400 AA Deventer, the Netherlands

This thesis

Abstract

Histomonosis is a parasitic disease caused by *Histomonas meleagridis* and occurs in turkeys and other (galliform) birds. It may result in very high mortality. Since the ban on effective chemotherapeutics that were successfully used in the past, turkey producers were left without any means for prevention or treatment. Therefore, there is urgent need for other chemotherapeutics. For both paromomycin and tiamulin activity against histomonosis has been reported previously. In the present study these antibiotics were examined *in vitro* by adding two-fold serial dilutions ranging from 12.5 to 400 µg/ml to cultures of *H. meleagridis*. Both negative and positive controls (12.5 µg and 400 µg/ml dimetridazol) were included. Parasites were counted after 3, 20, 28, 44, 51 and 71 hours of incubation. For tiamulin no clear antihistomonal effect was found, however, for paromomycin an inhibitory effect was found for all concentrations tested.

Paromomycin was subsequently examined in an *in vivo* study. Five groups of 20 one-day-old poults, matched by weight and sex were either untreated (positive and negative control group) or treated with 100, 200, or 400 ppm paromomycin. After two weeks all groups, except for the negative control group, were intracloacally inoculated with 200.000 histomonads per bird.

A clear dose-response effect was found for paromomycin. In the 100 ppm paromomycin mortality was similar to the positive control group, in the 200 ppm paromomycin approximately half of the turkeys died, while in the 400 ppm paromomycin group almost complete protection against histomonosis was seen. In the 200 ppm paromomycin a marked difference in susceptibility between sexes was observed.

Introduction

Histomonosis is a re-emerging disease in commercial poultry. It is caused by a flagellated protozoan, *Histomonas meleagridis*. In the 1960's several chemotherapeutics were developed that were highly effective to control histomonosis. Especially nitroimidazoles (e.g. dimetridazole) and arsenical compounds (e.g. nitarsone) have been widely and successfully applied in commercial poultry (McDougald, 2005). Increasing concern for the presence of residues in food and possible toxicity and carcinogeneticy of these therapeutics led to their ban in Europe some years ago (McDougald, 2005). This left turkey and chicken breeders without effective treatment against histomonosis. In recent years alternative products, like feed additives based on herbs, have been screened for a possible antihistomonal effect in order to provide the poultry industry with an effective treatment against this parasite. Both positive and negative results were obtained during *in vitro* (Van der Heijden and Landman, 2008b) and *in vivo* studies on herbal extracts (Duffy et al., 2005; Hafez and Hauck, 2006; Van der Heijden and Landman, 2008a), therefore their antihistomonal effect remains controversial.

Paromomycin is an antibiotic belonging to the family of aminoglycosides. Its mechanism of action is based on a RNA-binding capacity (Schroeder et al., 2000). Paromomycin has mainly been used for the treatment of leishmaniasis (Loiseau and Bories, 2006), but has also been found to be effective against *Dientamoeba fragilis* (Chan et al., 1994; Vandenberg et al., 2007), an intestinal protozoan parasite causing gastrointestinal disease in humans, that is both morphological and phylogenetically very closely related to *H. meleagridis* (Gerbod et al., 2001). In only two studies paromomycin was tested for a possible antihistomonal effect (Hu and McDougald, 2004; Lindquist, 1962), with opposite findings.

Another antibiotic, tiamulin, was recently reported to have an antihistomonal effect in a case study (Burch et al., 2007), in which the reduced mortality in a turkey flock suffering from an outbreak of histomonosis was attributed to medication with this drug. However, as this study did not include untreated controls, the reduced mortality found may have been coincidental.

In the present study, both paromomycin and tiamulin were first examined for their possible antihistomonal effect using an *in vitro* test (Van der Heijden and Landman, 2008b). Since paromomycin, unlike tiamulin, was found to have an antihistomonal *in vitro* effect it was further examined in an *in vivo* model (Van der Heijden and Landman, 2008a).

Material & Methods

***In vitro* study**. *H. meleagridis* isolate Turkey/Deventer/NL/AL327-type I/03 (Van der Heijden et al., 2006) was isolated from a turkey with typical liver and cecal

histomonal lesions (Van der Heijden et al., 2005). The isolate was resuscitated from liquid nitrogen storage by quickly thawing and inoculating a 1 ml aliquot into 12.5 ml of pre-warmed Dwyer's medium (Dwyer, 1970), consisting of 85% Medium 199 with Hank's salts (Gibco-BRL), 10% heat-inactivated horse serum (Gibco-BRL), 5% chicken embryo homogenate of 8 day-old eggs and 12 mg of rice powder (Arrowhead Mills, Hereford, ISA). After an incubation period of 3 days at 40 °C subculturing was done by inoculation of 1 ml of culture each into five tissue culture flasks with 12.5 ml of modified Dwyer's medium (Van der Heijden and Landman, 2007), consisting of 90% Medium 199, 10% horse serum and 100 mg of rice powder. After 24 hours of incubation the contents of the five culture flasks were pooled. The concentration of parasites of the pooled culture was determined by a haemotocytometer and microscopy at 200x magnification.

Fresh 10 mg/ml stocks of paromomycin sulphate (Biovet, Peshtera, Bulgaria), tiamulin hydrogen fumarate (Biovet) and dimetridazole (Sigma-Aldrich, USA) in Phosphate Buffered Saline (PBS) were prepared by dissolving 100 mg of product in 10 ml PBS. The solution was thoroughly vortexed until the compounds were completely dissolved. From these stock suspensions two-fold serial dilutions in PBS were prepared, corresponding with end concentrations in culture ranging from 2.5 to 400 µg/ml. Dimetridazole was tested as a positive control at 12.5 and 400 µg/ml only (in duplicate), and PBS was tested in duplicate as a negative control.

The histomonad test cultures were prepared by pre-warming new tissue culture flasks containing 100 mg rice powder, 8.6 ml of M199 and 950 µL of horse serum at 40°C. Subsequently 521 µL of a product solution was pipetted into the tissue culture flask and mixed with the medium by gently swirling. Finally, 3 ml of the pooled *H. meleagridis* culture was added. The cultures were incubated at 40 °C. The concentration of parasites in each flask was determined by the counting technique described above after 3, 20, 28, 44, 51 and 71 hours of incubation.

***In vivo* study**. In an experimental room (6.85 m x 4.48 m), which was only accessible for qualified personnel after changing of footwear and overall clothing, five stainless steel wire pens (1.3 m x 1 m) were assembled. The pens were positioned at a distance of 40 cm from each other, with the side walls covered with plastic to minimize to risk of cross infections between groups. Approximately 8 cm of wood shavings was used as bedding.

One-day-old BUT Big 6 poults (approximately 50% female/50% male) were tagged (Swittack®) and individually weighed. Subsequently, they were distributed equally to the experimental groups in order to avoid differences in average weight between the groups. The lighting program consisted of 2 h of darkness and 22 h of light during the first week, and 8 h of darkness and 16 h of light for the next five weeks until the end of the experiment. Groups 1 and 2 received non-medicated turkey feed, while the groups 3 to 5 received turkey feed with 100, 200, or 400 ppm paromomycin, respectively. Both feed and drinking water were supplied *ad libitum*.

At two weeks of age, the turkeys of all experimental groups, except group 2, were intracloacally inoculated with *H. meleagridis* strain /Deventer/NL/AL327-type I/ (Van der Heijden et al., 2006) using an inoculation dose of 200.000 histomonads per bird, as described elsewhere (Van der Heijden and Landman, 2008a). The control group 2 was inoculated with culture medium without histomonads. Throughout the experiment (feed supply, weighing, removal of dead birds from the pens, etc.) the negative control group birds were always handled last in order to detect possible cross-infections. After inoculation, the birds were inspected twice daily and dead birds removed from the pens. Birds that were very sick and did not eat for two consecutive inspections were euthanized by intravenous injection with T61 (Intervet, Boxmeer, the Netherlands). The poults were individually weighed weekly. After six weeks the remaining birds were subjected to postmortem analysis. Birds were stunned using CO_2 plus O_2 and exsanguinated. Birds that died during the experiment were also necropsied. Liver and caecal lesion were scored on a scale ranging from 0 to 4 (McDougald and Hu, 2001).

Statistical analysis. The time of survival was compared using the non-parametrical Kruskall-Wallis comparison test. Pairwise two-samples survival testing was done using the non-parametric Gehan-Wilcoxon test. Statistical analyses were performed using Statistix 8 (Analytical Software, Tallahassee, USA).

Results

***In vitro* study**. The concentration of the pooled culture was 2.3×10^6 histomonads per ml; i.e. the starting concentration in the test cultures was 0.55×10^6 histomonads/ml.

Compared with the negative control cultures, the addition of dimetridazole at a concentration of 400 µg/ml resulted already within 3 hours in lower parasite concentrations (Figure 1), while after 20 hours the numbers of parasites had dropped below the detection limit. In cultures with dimetridazole at a concentration of 12.5 µg/ml tested in duplicate, the number of histomonads also gradually declined over time, and was below the detection limit after 44 or 51 hours. In the negative control cultures the number of histomonads increased between 3 and 28 hours of incubation and then slightly decreased during the rest of the experiment.
In comparison with the negative control experiment (Figure 1), addition of paromomycin (Figure 2) to the *H. meleagridis* cultures resulted in a gradual decrease in histomonad counts after 28 hours of incubation being below or close to the detection limit at the end of the experiment. Although the culture with the lowest concentration of paromomycin tested (12.5 µg/ml) had the highest parasite counts at the end of the experiment, and the culture with the highest concentration tested

(400 µg/ml) had the lowest parasite numbers after 28 hours and later, the results of all concentrations tested were similar.

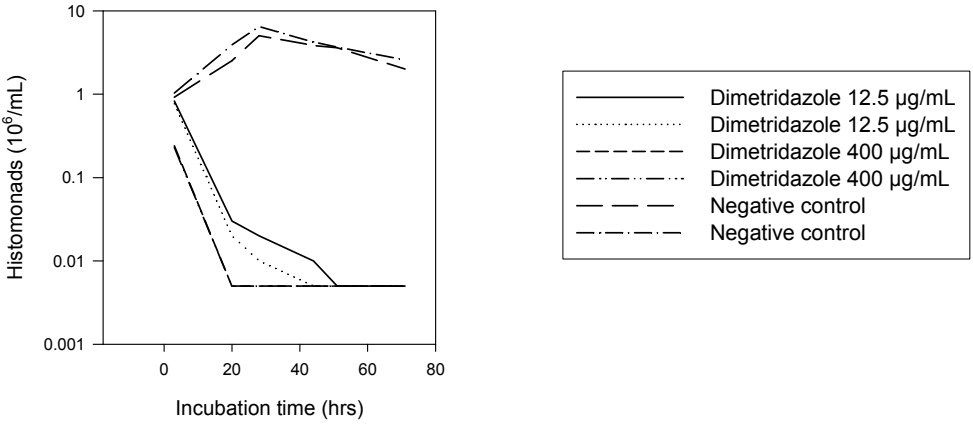


Figure 1: Histomonad counts of cultures with Dimetridazole at 12.5 or 400 µg/ml, or without treatment (negative control).

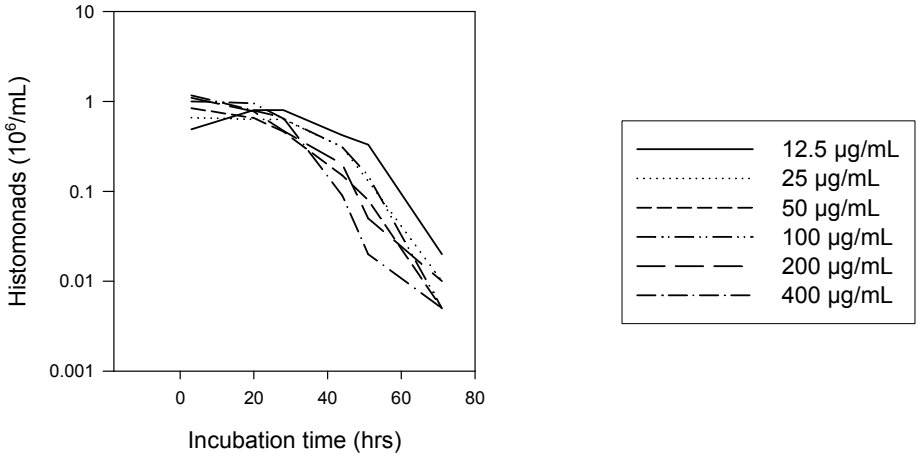


Figure 2: Histomonad counts of cultures with serial dilutions of paromomycin.

In comparison with the negative control cultures (Figure 1) only slightly lower numbers of histomonads were found in the cultures with tiamulin (Figure 3). In the

culture with the highest concentration of tiamulin tested (400 µg/ml), after 28 hours of incubation until the end of the experiment, parasite counts were only slightly lower than in the negative control cultures.

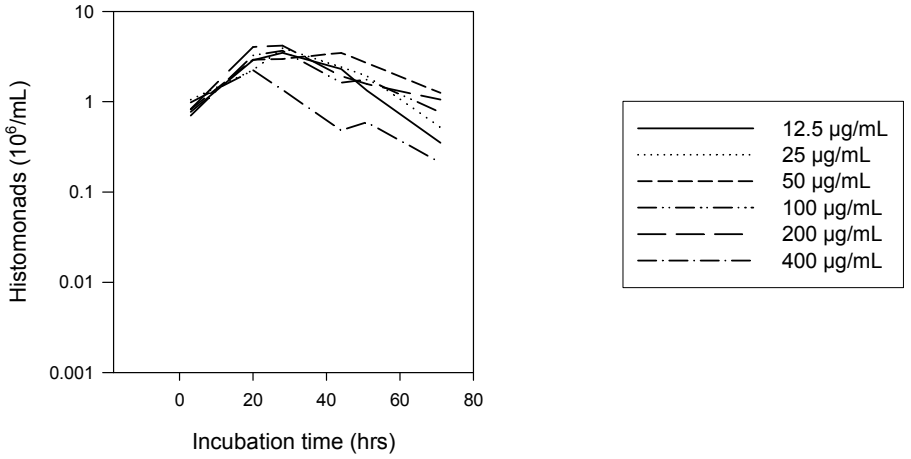


Figure 3: Histomonad counts of cultures with serial dilutions of tiamulin.

***In vivo* study**. In the negative control group (no treatment, not inoculated) one turkey had to be euthanized at 8 days post inoculation due to a slipped tendon. Upon postmortem examination no signs indicative of histomonosis were seen, as ceca and liver did not show macroscopic lesions. All remaining turkeys of this group survived until the end of the experiment and did not show liver, cecal or other macroscopic lesions at postmortem.

In the positive control group (no treatment, inoculated) and the experimental group treated with 100 ppm paromomycin the first birds died of histomonosis (lesions in ceca an livers) at 11 days p.i. (Figure 4). Around 15 days p.i. only half of the turkeys in these groups had survived, and after 22 days p.i. only two, and one bird survived in the positive control group, and the 100 ppm paromomycin group, respectively.

In the experimental group treated with 200 ppm paromomycin, the first turkey died 14 days p.i.. After 25 days p.i. half of the birds had survived. At the end of the experiment 45% of the turkeys were still alive.

All birds that died or were euthanized during the experiment because of illness, except for a single turkey in the negative control group, had clear signs of histomonosis upon necropsy, with cecal and liver lesions scores (± standard deviation) averaging 3.8 ± 0.46 for cecal lesions and 3.2 ± 0.60 for liver lesions.

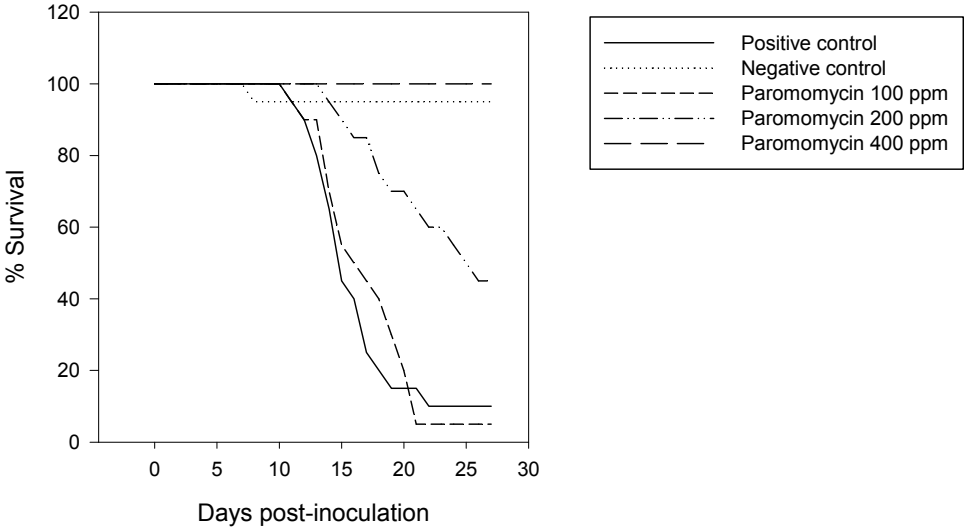


Figure 4: Survival of turkeys in different experimental groups following inoculation with *H. meleagridis*.

In the experimental group treated with 400 ppm paromomycin, not a single turkey died throughout the experiment. At postmortem a single (female) bird had lesions in the ceca (score 3), but a normal liver. All other turkeys in this group had completely normal ceca and livers.

Pairwise two-samples survival analysis showed that the 100 ppm paromomycin group was not significantly ($P>0.05$) different from the positive control group, in contrast with the 200 and 400 ppm paromomycin group ($P<0.01$). On the other hand, the 400 ppm paromomycin group was not significantly ($P>0.05$) different from the negative control group, while 200 ppm and 100 ppm groups were significantly different ($P<0.01$) from the negative control group.

In Table 1 the results of mortality of female and male turkeys are presented separately. The treatment had a significant effect (Kruskall-Wallis, $P<0.001$) on the day of survival for both sexes. In the group treated with 100 ppm paromomycin for both sexes the mortality was similar to the positive control group and the birds on average did not die significantly later. In the group treated with 200 ppm paromomycin all male turkeys did not die significantly later in comparison with the positive control group. In contrast most female turkeys in this group survived, and the day of survival was not significantly different from the negative control group. Pairwise survival analysis confirmed the difference between the female and male

birds (P<0.01) in this group. In the group treated with 400 ppm paromomycin all turkeys survived.

Table 1: The number of female or male turkeys that died and the average number of days of survival in experimental groups with different treatments.

	Female		Male	
Experimental group	# died	days survival	# died	days survival
Positive control	10/10	14.8 ± 1.75^{A}	8/10	18.5 ± 5.87^{A}
Negative control	0/9	28.0 ± 0.00^{B}	1*/11	26.2 ± 6.03^{BC}
100 ppm paromomycin	10/10	15.3 ± 2.91^{A}	9/10	19.1 ± 4.18^{AB}
200 ppm paromomycin	2/11	27.2 ± 1.94^{B}	9/9	19.0 ± 4.03^{A}
400 ppm paromomycin	0/11	28.0 ± 0.00^{B}	0/9	28.0 ± 0.00^{C}

Means with identical superscripts are not significantly (P<0.05) different among sexes.
* bird euthanized due to slipped tendon.

The addition of paromomycin had no adverse effect on feed intake. In the negative control group the average feed consumption for the experiment was 115 g per bird per day, while for the group receiving 400 ppm the average feed consumption was 122 g per bird per day.

The weekly average weight of the female turkeys (Figure 5a) was approximately the same for the negative control groups and the groups treated with 200 and 400 ppm paromomycin. In the positive control group and the group receiving 100 ppm paromomycin in their feed, the birds did not increase in weight after one week p.i. In the male turkeys (Figure 5b), after inoculation, the average weekly weight of the group treated with 200 ppm paromomycin was less than the average weekly weight of the control group and the group receiving 400 ppm paromomycin. In the positive control group the increase in weight of the turkeys in the positive control group was less and similar to the birds receiving 100 ppm paromomycin. The sudden increase in average weight in the positive control group towards the end of the experiment is caused by the fact that one of the two surviving birds grew much better than the other turkeys (Table 2).

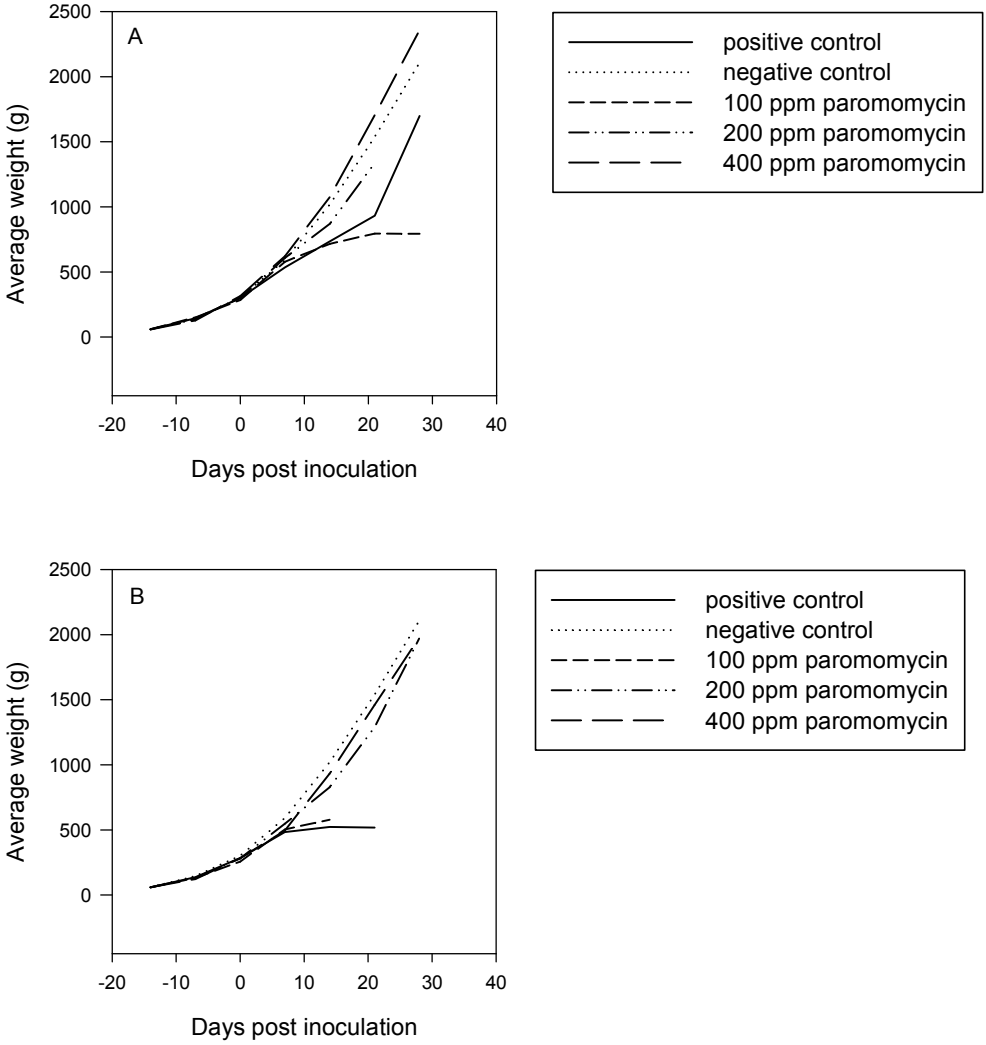


Figure 5: Average weekly weight of female (A) or male (B) turkeys in groups with different treatments and experimental inoculation with *H. meleagridis*.

Table 2: Average weight (g) of turkeys ± standard error and number of birds in experimental groups with different treatments against histomonosis.

Age	Positive control		Negative control		100 ppm paromomycin		200 ppm paromomycin		400 ppm paromomycin	
(wks)	female	male	female	male	female	male	female	male	female	male
0	59 ± 1.4 (n =10)	59 ± 1.8 (n=10)	59 ± 1.7 (n=9)	58 ± 1.6 (n=11)	58 ± 1.1 (n=10)	60 ± 1.2 (n=10)	59 ± 1.1 (n=11)	60 ± 1.1 (n=9)	59 ± 1.1 (n=11)	58 ± 1.7 (n=9)
1	136 ± 6.4 (n=10)	144 ± 7.6 (n=10)	132 ± 10.2 (n=9)	145 ± 5.6 (n=11)	132 ± 5.8 (n=10)	150 ± 4.8 (n=10)	133 ± 7.4 (n=11)	137 ± 11.4 (n=9)	123 ± 5.7 (n=11)	128 ± 6.8 (n=9)
2	286 ± 11.2 (n=10)	301 ± 15.8 (n=(10)	265 ± 21.3 (n=9)	301 ± 13.6 (n=11)	257 ± 11.3 (n=10)	286 ± 8.0 (n=10)	281 ± 11.9 (n=11)	300 ± 20.0 (n=9)	283 ± 10.3 (n=11)	315 ± 15.7 (n=9)
3	484 ± 13.1 (n=10)	535 ± 30.4 (n=10)	510 ± 33.2 (n=9)	591 ± 22.4 (n=11)	504 ± 19.4 (n=10)	578 ± 16.2 (n=10)	547 ± 18.9 (n=11)	607 ± 35.4 (n=9)	495 ± 44.8 (n=11)	621 ± 32.6 (n=9)
4	524 ± 18.0 (n=6)	736 ± 88.4 (n=7)	870 ± 49.5 (n=9)	1019 ± 35 (n=10)	578 ± 40.0 (n=6)	716 ± 42.7 (n=8)	829 ± 83.9 (n=11)	871 ± 84.6 (n=8)	931 ± 23.4 (n=11)	1075 ± 40 (n=9)
5	518 ± 42.0 (n=2)	932 ± 93.2 (n=4)	1312 ± 69.6 (n=9)	1537 ± 47 (n=10)	- (n=0)	794 (n=1)	1288 ± 115 (n=11)	1337 ± 27 (n=2)	1459 ± 32.5 (n=11)	1704 ± 57 (n=9)
6	- (n=0)	1697 ± 901 (n-2)	1795 ± 89.7 (n=9)	2107 ± 61 (n=10)	- (n=0)	792 (n=1)	1970 ± 152 (n=9)	- (n=0)	1977 ± 33.4 (n=11)	2357 ± 71 (n=9)

Discussion

In the *in vitro* study, the results of both the positive control (dimetridazole) and the negative control (no treatment) where in agreement with those of previous experiments (Van der Heijden and Landman, 2008b) and literature (Callait et al., 2002; Hu and McDougald, 2004).

In a recent case study (Burch et al., 2007) mortality in a turkey flock suffering from an outbreak of *H. meleagridis* quickly dropped from 12 to 13 birds per day to 3 to 4 birds per day after receiving 25 mg tiamulin/kg bodyweight via drinking water. As untreated control birds were not included in mentioned study, the decline of mortality may have been coincidental and not the consequence of tiamulin treatment. This is further evidenced by the fact that mortality in the herd already had been declining before the start of the treatment. Nevertheless, as an antihistomonal effect of tiamulin could not be excluded, it was tested in an *in vivo* experiment. As no clear *in vitro* antihistomonal effect was found, it is not very likely that tiamulin would have an *in vivo* antihistomonal effect, and it was therefore not further examined.

Paromomycin is an antibiotic that is effective against *D. fragilis* (Chan et al., 1994; Vandenberg et al., 2007), which is very closely related to *H. meleagridis*. Therefore, it was considered worthwhile to examine it for a possible antihistomonal effect.

During the *in vitro* experiment a clear antihistomonal effect was found for paromomycin. However, a clear dose-response effect could not be detected for the concentrations tested. Therefore, it seems plausible that the *in vitro* minimal lethal dose (MLD) is probably lower than 12.5 µg/ml. At the highest concentration tested, the inhibitory effect of paromomycin was less than that of dimetridazole at 12.5 µg/ml.

H. meleagridis is dependent on the presence of cecal bacteria in the culture (dixenic culture), and a bactericidal effect might be responsible for a false positive result (antihistomonal effect). In the present study none of the treatments resulted in a less turbid culture (i.e. complete inhibition of bacterial growth). But as several species of bacteria are present in the culture, it remains uncertain whether a certain species is eliminated that is essential for histomonal growth *in vitro*. Therefore, positive *in vitro* results always should be confirmed by an *in vivo* study.

Paromomycin also showed a clear antihistomonal effect in the *in vivo* study. In addition a clear dose-response effect was observed, with no protection against histomonosis at 100 ppm, a partly effect at 200 ppm and almost complete protection at 400 ppm paromomycin. Lesion scores were not statistically compared between groups, because birds that died of histomonosis during the study always had high scores, while surviving birds had no lesions. Differences in lesions scores would therefore depend on differences in mortality rather than on a direct effect of paromomycin on the development of lesions. Although little is known about the

relative virulence of *H. meleagridis* non-attenuated strains in general, it is likely that the isolate used in the present study has a high virulence, since in a previous study (Van der Heijden and Landman, 2008a) Histostat-50™ (nitarsone) as a feed additive at 375 ppm hardly showed any antihistomonal effect, and even moderate mortality and morbidity was seen in the control group treated with 200 ppm dimetridazole. Lindquist (Lindquist, 1962) also found high (70%) protection against histomonosis after treatment of turkeys with very high levels (1000 to 2000 ppm) of paromomycin. In a recent study (Hu and McDougald, 2002), although a complete inhibitory effect of paromomycin was obtained *in vitro*, paromomycin at 200 or 400 ppm gave no protection *in vivo*. However, it should be noted that this study was performed in chickens. Besides different species of birds, there are other dissimilarities between these experiments. Inoculation was either done using embryonated eggs of *H. gallinarum* infected with *H. meleagridis* (Lindquist, 1962), using a co-infection of *H. meleagridis* and *Eimeria tenella* (Hu and McDougald, 2004) or only *H. meleagridis* in this study. Another possible important difference between the studies is the period of treatment before infection. This ranged from one day (Hu and McDougald, 2004), 4 to 8 days (Lindquist, 1962), to 14 days of the present study. These findings suggest that possibly long exposure to the antibiotic in feed is necessary in order to detect an antihistomonal effect.

A remarkable finding was the difference between mortality in female and male turkeys in the group treated with 200 ppm paromomycin. This is in contrast with previous findings in both chickens (Chute et al., 1976) and turkeys (Liebhart et al., 2008; Van der Heijden and Landman, 2008a). The somewhat higher body weight of the males does not mean that the male birds were treated with a slightly lower dosage of paromomycin per gram of body weight, since also their feed intake was proportionally higher. At present, a plausible explanation for the difference in mortality between the male and female birds in the 200 ppm paromomycin group is lacking.

Paromomycin had no negative effect on either average feed intake, or on average weekly growth of the turkeys. The growth curves of both female and male birds receiving feed with 400 ppm paromomycin were very similar to the growth curves of the negative control group.

References

Burch, D., Young, S., Watson, E., 2007, Treatment of histomonosis in turkeys with tiamulin. Veterinary Record 161, 864.

Callait, M.P., Granier, C., Chauve, C., Zenner, L., 2002, *In vitro* activity of therapeutic drugs against *Histomonas meleagridis* (Smith, 1895). Poultry Science 81, 1122-1127.

Chan, F.T., Guan, M.X., Mackenzie, A.M., Diaz-Mitoma, F., 1994, Susceptibility testing of *Dientamoeba fragilis* ATCC 30948 with iodoquinol, paromomycin, tetracycline, and metronidazole. Antimicrobial Agents and Chemotherapy 38, 1157-1160.

Chute, A.M., Lund, E.E., Wilkins, G.C., 1976, Comparative responses of white leghorn and New Hampshire chickens to experimental infections with *Histomonas meleagridis* and *Heterakis gallinarum*. Poultry Science 55, 710-713.

Duffy, C.F., Sims, M.D., Power, R.F., 2005, Evaluation of dietary Natustat for control of *Histomonas meleagridis* in male turkeys on infected litter. Avian Diseases 49, 423-425.

Dwyer, D.M., 1970, An improved method for cultivating *Histomonas meleagridis*. Journal of Parasitology 56, 191-192.

Gerbod, D., Edgcomb, V.P., Noel, C., Zenner, L., Wintjens, R., Delgado-Viscogliosi, P., Holder, M.E., Sogin, M.L., Viscogliosi, E., 2001, Phylogenetic position of the trichomonad parasite of turkeys, *Histomonas meleagridis* (Smith) Tyzzer, inferred from small subunit rRNA sequence. Journal of Eukaryotic Microbiology 48, 498-504.

Hafez, H.M., Hauck, R., 2006, Efficacy of a herbal product against *Histomonas meleagridis* after experimental infection of turkey poults. Archives of Animal Nutrition 60, 436-442.

Hu, J., McDougald, L.R., 2002, Effect of anticoccidials and antibiotics on the control of blackhead disease in broiler breeder pullets. Journal of Applied Poultry Research 11, 351-357.

Hu, J., McDougald, L.R., 2004, The efficacy of some drugs with known antiprotozoal activity against *Histomonas meleagridis* in chickens. Veterinary Parasitology 121, 233-238.

Liebhart, D., Graybill, H.W., Hess, M., 2008, A virulent mono-eukaryotic culture of *Histomonas meleagridis* is capable of inducing fatal histomonosis in different aged turkeys of both sexes, regardless of the infective dose. Avian Diseases 52, 168-172.

Lindquist, W.D., 1962, Some effects of paromomycin sulfate on blackhead in turkeys. American Journal of Veterinary Research 23, 1053-1056.

Loiseau, P.M., Bories, C., 2006, Mechanisms of drug action and drug resistance in *Leishmania* as basis for therapeutic target identification and design of antileishmanial modulators. Current Topics in Medical Chemistry 6, 539-550.

McDougald, L.R., 2005, Blackhead disease (histomoniasis) in poultry: a critical review. Avian Diseases 49, 462-476.

McDougald, L.R., Hu, J., 2001, Blackhead disease (*Histomonas meleagridis*) aggravated in broiler chickens by concurrent infection with cecal coccidiosis (*Eimeria tenella*). Avian Diseases 45, 307-312.

Schroeder, R., Waldsich, C., Wank, H., 2000, Modulation of RNA function by aminoglycoside antibiotics. EMBO Journal 19, 1-9.

Van der Heijden, H.M.J.F., Landman, W.J., 2007, Improved culture of *Histomonas meleagridis* in a modification of Dwyer medium. Avian Diseases 51, 986-988.

Van der Heijden, H.M.J.F., Landman, W.J., 2008a, *In vivo* effect of herbal products against *Histomonas meleagridis* in turkeys. Avian Pathology 37, 45-50.

Van der Heijden, H.M.J.F., Landman, W.J.M., 2008b, *In vitro* effect of herbal products against *Histomonas meleagridis*. Veterinary Parasitology 154, 1-7.

Van der Heijden, H.M.J.F. Landman, W.J., Greve, S., Peek, R., 2006, Genotyping of *Histomonas meleagridis* isolates based on internal transcribed spacer-1 sequences. Avian Pathology 35, 330-334.

Van der Heijden, H.M.J.F., McDougald, L.R., Landman, W.J., 2005, High yield of parasites and prolonged *in vitro* culture of *Histomonas meleagridis*. Avian Pathology 34, 505-508.

Vandenberg, O., Souayah, H., Mouchet, F., Dediste, A., van Gool, T., 2007, Treatment of *Dientamoeba fragilis* infection with paromomycin. Pediatrics Infectious Disease Journal 26, 88-90.

Chapter 4

Development and validation of a blocking ELISA for the detection of antibodies against *Histomonas meleagridis* in chickens and turkeys

H.M.J.F. van der Heijden[1], J.A. Stegeman[2] and W.J.M. Landman[1]

1. Animal Health Service Ltd, P.O. Box 9, 7400 AA Deventer, the Netherlands
2. Utrecht University, Faculty of Veterinary Medicine, Department of Farm Animal Health, Yalelaan 7, 3584 CL Utrecht, the Netherlands

Submitted to Veterinary Parasitology

Abstract

Histomonosis is an infectious disease of mainly galliform birds that can cause high mortality, especially in commercial turkey flocks. A number of diagnostic and laboratory tools to identify the disease exist, however, a serological tool is lacking. Therefore, a specific blocking-ELISA for the detection of antibodies against its causative agent, *Histomonas meleagridis*, a flagellated protozoan was developed. Monoclonal antibodies were raised against a detergent extracted protein of *H. meleagridis*. The MAbs bound to morphologically identified histomonads in liver tissue of an infected turkey. After conjugation with horseradish peroxidase, the most promising MAb was selected for use as a conjugate in the blocking-ELISA. Experimentally infected turkeys (n=9) and chickens (n=10) seroconverted in the blocking-ELISA within 2 to 4 weeks after inoculation with a *H. meleagridis* field strain. The *H. meleagridis* blocking-ELISA did not cross-react with the closely related protozoan *Tetratrichomonas gallinarum*. The MAb did not bind to *T. gallinarum* antigen, moreover, *T. gallinarum* seropositive layer chickens (n=18) showed the same inhibition percentages as negative control birds. The repeatability and reproducibility of the *H. meleagridis* blocking-ELISA were high.

Introduction

Histomonas meleagridis is a flagellated protozoan (phylum Parabasalida, class Trichomonadea, family Monocercomonadidae) that may cause histomonosis (infectious enterohepatitis, blackhead disease) mainly in galliform birds, especially turkeys, and sporadically in other species of birds. In turkey flocks, morbidity and mortality can be as high as 100% (McDougald, 1998). Nitroheterocyclic compounds like dimetridazole and arsenical compounds like nitarsone have been successfully used as prophylactic and curative therapies. But in recent years all drugs with known antihistomonal activity have been banned from use in production birds in Europe, while in the US only nitarsone is allowed (McDougald, 2005). The ban of these products resulted from concerns about possible toxic and carcinogenic properties. As a consequence histomonosis has become a re-emerging disease and the need to develop new strategies for the prevention (e.g. biosecurity, vaccination) and treatment (new products) of histomonosis grew parallely. In order to fulfil these needs, histomonas research was boosted. In the search of new antihistomonal strategies, animal models a number of existing laboratory tools have been used, however the possibility of serological analysis was lacking. Serology could prove useful: 1) to detect seroconversions in transmission studies examining new antihistomonal products, which is probably a more sensitive indicator of transmission than morbidity and mortality, or 2) to perform seroepidemiological studies. Chickens are considered to be a reservoir for the parasite (McDougald, 2005), but to what extent commercial chicken flocks are carriers of histomonads is yet unknown. Without a serological tool it would be difficulty and expensive to determine the prevalence in chicken flocks, while a seroprevalence study using an ELISA is relatively simple and cost-effective.

Little is known about the humoral response of turkey and chicken against *H. meleagridis*. Clarkson (1963) used a double diffusion method and found that both species of birds developed precipitating antibodies following experimental infection. The antibodies could be detected after 14 and 12 days post infection (dpi) and were still present after 65 and 83 dpi in turkeys and chicken, respectively. Polyclonal antibodies were raised against *H. meleagridis* in rabbits for 1) antigen detection purposes by IFT (Hall and Krishnam, 1967) or immunohistochemistry (Singh et al., 2008), 2) to study the possibility of using an attenuated *H. meleagridis* strain (Dwyer, 1971; Dwyer and Honigberg, 1972) or non-pathogenic *Parahistomonas wenrichi* (Augustine and Lund, 1968, 1970) as a vaccine, and 3) to examine the antigenic relationship among *Histomonas*, *Trichomonas*, *Dientamoeba* and *Entamoeba* (Dwyer, 1972a, b, 1974). Apart from a close antigenic relation between *H. meleagridis* and the human parasite *Dientamoeba fragilis*, also strong cross-reactivity was observed between *H. meleagridis* and *Trichomonas gallinarum*, nowadays known as *Tetratrichomonas gallinarum*, and which is common in galliform and anseriform birds (Cepicka et al., 2005). As a

consequence, it was quite likely that an indirect ELISA would suffer from cross-reactivity with *T. gallinarum*. Therefore, in the present study a specific monoclonal antibody (MAb) against *H. meleagridis* was produced and used to develop a specific blocking-ELISA for detection of turkey and chicken antibodies against *H. meleagridis*. To our knowledge, this is the first report about a *H. meleagridis* MAb as well as the first article describing a *H. meleagridis* ELISA.

Material & Methods

***H. meleagridis* antigen**. *H. meleagridis* strain /Deventer/NL/AL327-type I/03 (Van der Heijden et al., 2006), a cryopreserved pathogenic Dutch *H. meleagridis* field isolate originating from turkeys, was resuscitated in Dwyer's medium (Dwyer, 1970) by inoculating five 1 ml aliquots each into 12.5 ml of medium pre-warmed at 40 °C. In addition, a control culture (consisting of bacterial flora and medium ingredients) that was obtained by killing the histomonads through a freeze-thaw step, was included. After 72 hours of incubation at 40 °C, the *H. meleagridis* cultures were pooled and subcultured in 45 tissue culture bottles, each by inoculating 1 ml of culture into 12.5 ml of pre-warmed modified Dwyer's medium (Van der Heijden and Landman, 2007). The control culture was subcultured in 5 bottles. After 48 hours of incubation the cultures were sieved through a nylon cloth filter of 100 µm (Stokvis & Smits, Almere, the Netherlands) to remove excess of rice particles, and pooled. The histomonads were counted using a haemocytometer and light microscopy. The 600 ml of pooled *H. meleagridis* culture with 3.3 million parasites per ml and 60 ml of control culture (no histomonads present) were centrifuged during 20 min at 200 x*g* at room temperature in a desktop centrifuge (Sigma laboratory centrifuges, Osterode am Harz, Germany). Pellets were washed once with 200 ml of Phosphate Buffered Saline (PBS) prewarmed at 40 °C, resuspended in 21 ml of warm PBS, and aliquoted in 3 ml portions. These were used to produce both crude *H. meleagridis* and control antigen as well as detergent-extracted *H. meleagridis* and control antigens.

Crude antigens were prepared by a freeze-thaw step followed by centrifugation during 10 minutes at 1000 x*g* to remove insoluble particles. The supernatants were aliquoted in 750 µl portions and stored at -20 °C until used.

Detergent extracted antigens were produced by the following procedure (Hemphill and Gottstein, 1996): phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, Zwijndrecht, the Netherlands) dissolved in isopropanol (Merck, Darmstadt, Germany) was added to a concentration of 0.2 mM. After 5 min of incubation on melting ice, 0.75% Triton-X 114 (Pierce, Rockford, USA) was added, and the suspensions gently vortexed. After 10 further min of incubation on melting ice, the suspension was centrifuged during 30 min at 10 000 x*g* at 4 °C in a high speed centrifuge (Beckman-Coulter, Mijdrecht, the Netherlands). The supernatant was

collected in a clean tube and incubated for 3 min at 30 °C, cooled on melting ice during 10 min, and centrifuged again (30 min; 10 000 x*g*; 4 °C) to remove remaining aggregates. The supernatant was collected in a clean tube and incubated again during 3 min at 30 °C. The detergent phase was separated from the water phase by centrifugation during 3 min at 1000 x*g* at 24 °C in the desktop centrifuge. The water phase was discarded.

Protein was purified from the detergent phase using a quantitative recovery method (Wessel and Flugge, 1984). To 100 µl of the detergent phase containing proteins, 400 µl methanol (Merck) was added. After vortexing and centrifugation at 9000 x*g* for 10 sec, 100 µl chloroform (Merck) was added. After further vortexing and centrifugation at 9000 x*g* for 10 sec, 300 µl sterile demineralised water was added. The suspension was vigorously vortexed. Following centrifugation during 1 min at 9000 x*g* the upper phase was carefully collected and discarded. Then, 300 µl of methanol was added to the chloroform phase. After further vortexing and centrifugation for 2 min at 9000 x*g* the supernatant was removed and the protein pellet dried under a stream of air. After resuspension in PBS the protein concentrations were determined by measurement of the absorbance at a wavelength of 280 nm, after which the protein concentration was adjusted to 250 µg/ml.

Monoclonal antibodies. Five female Balb/c mice of about 10 wks old were immunized three times with 50 µg of the *H. meleagridis* antigen formulated in Stimune (Prionics, Lelystad, the Netherlands). At day 39, four days after the last immunization, spleen cells were fused to SP2/0 cells using a protocol in which PEG (polyethylene glycol) was used in the fusion medium to facilitate fusion of adjacent plasma membranes. As the myeloma cells do not have the ability to synthesize hypoxanthine-guanine- phosphoribosyltransferase, fused cells were selected in HAT (hypoxantine, amenopterin and thymidin) in Iscove's Modified Dulbecco's Medium with glutamax (IMDM; Invitrogen Live Technologies, Carlsbad, USA) supplemented with 1% Hybridoma Enhancing Supplement (Invitrogen). Culture supernatants were tested in an indirect *H. meleagridis* ELISA for specific antibody production. Positive clones were sub-cloned twice to reach a monoclonal cell suspension.

The immunoglobulin production of five hybridomas was scaled up using a disposable bioreactor system (CELline™, Integra Biosciences, Chur, Switzerland) according to the instructions of the producer. Briefly, after prewetting the membrane with medium, in the cell suspension compartment 1 ml of hybridoma cell suspension was added to 5 ml of culture medium, consisting of IMDM medium, 10% fetal calf serum (Hyclone; Perbio Science, Etten-Leur, the Netherlands), 2% Sodium Pyruvate (Sigma-Aldrich) and 1% of an antibiotic mixture consisting of 10 kU/ml penicillin, 10 mg/ml streptomycin and 25 µg/ml of amphotericin-B. The other compartment was filled with 340 ml of maintenance medium which is similar to the

culture medium but contains 1% instead of 10% fetal calf serum. The cell lines were cultured at 37 °C and passaged 7 to 11 times at weekly intervals. This involved harvesting part of the culture medium containing the immunoglobulins. The cell suspension was collected using a disposable 10 ml pipette and centrifuged for 10 minutes at 200 x*g* at room temperature to pellet the cells. 4 ml of supernatant was collected and stored at -20 °C. The pellet was resuspended in the remaining supernatant and 4 ml of fresh culture medium and returned to the cell compartment. The maintenance medium was refreshed.

Indirect ELISA's. A number of indirect ELISA's were developed using both crude antigens and detergent extracted antigens. Checkerboard titrations were performed to determine the optimal antigen coating concentrations as well as the conjugate dilutions. For the indirect chicken and turkey ELISA the conjugate used was a rabbit anti chicken IgG-HorseRadish Peroxidase (HRPO) conjugate (Nordic, Tilburg, the Netherlands), while for the indirect mouse ELISA a rabbit anti mouse-HRPO conjugate (Dakopatts, Glostrup, Denmark) was applied.

Briefly, the indirect ELISA procedures were as follows: antigen was diluted in PBS. 100 µl was added to the wells of a high binding ELISA plate (Costar, Badhoevedorp, the Netherlands), which was subsequently sealed and incubated overnight at ambient temperature. Plates were emptied and blocked using 200 µl of 1% Bovine Serum Albumin (BSA; Sigma-Aldrich) during 45 minutes at room temperature. The plates were washed 3 times with ultra pure water containing 0.05% tween 80 (Merck) and tapped empty. Sera were diluted 1/00 (chicken and turkey) or tested in a $\log_{10}$ fold serial dilution of 1/20 until 1/20 000 (mouse sera) or undiluted (hybridoma culture supernatants) in ELISA-buffer, consisting of PBS containing 0.05% tween 80, 0.1% BSA and 2% extra NaCl (Merck). 100 µl was added to the wells of the ELISA-plate, followed by incubation at room temperature for 1 h. Plates were washed again and 100 µl conjugate, diluted in ELISA-buffer, was added. Plates were again incubated for 1 h at room temperature and washed five times. Then, 100 µl of substrate solution, consisting of 0.005% H_2O_2 (Merck) in 0.10 M acetate/citric acid buffer pH 5.5 with 0.1 mg/ml of TMB chromogen (Fluka AG, Buchs, Switzerland), was added. The plates were incubated for 15 minutes at room temperature. The enzyme reaction was stopped by the addition of 50 µl 1 M sulphuric acid. Optical Densities (OD) were measured using an ELISA-reader (Tecan, Männedorf, Germany) at a wavelength of 450 nm.

Immunohistochemistry. Slides were cut from paraffin embedded liver tissues from turkeys infected with *H. meleagridis*. The slides were blocked with 10% Fetal Bovine Serum in PBS during 40 minutes at room temperature, incubated with monoclonal culture supernatant during 90 minutes at 37 °C, and rinsed three times with PBS containing 0.05% tween 20. Endogenic peroxidase was blocked by incubation with 1% H_2O_2 in methanol containing 0.1% NaN_3 during 20 minutes at

room temperature. After further rinsing the slides were incubated with a conjugate consisting of a dextran polymer conjugated with goat-anti-mouse-HRPO (Envision+ system, Dakopatts, Glostrup, Denmark) for 30 minutes at room temperature. After rinsing, the slides were developed using a DAB/H_2O_2 substrate solution (Dakoliquid DAB substrate chromogen system, Dakopatts) during 5 minutes at room temperature and rinsed with PBS. Finally, the slides were rinsed with tap water during 5 minutes and subsequently stained with haematoxylin.

Conjugation of Monoclonal Antibodies (MAb). For each of the 5 hybridoma's the weekly CELline™ culture medium harvests were tested in indirect ELISA to monitor antibody production with time. For each clone, 2 ml of the weekly harvests were pooled. The pools were centrifugated during 10 minutes at 2100 x*g* to remove cell rests.

Immunoglobulins were purified according to the instructions of the producer from the pooled culture media using the Melon™ monoclonal purification kit (Pierce) consisting of 100x stock purification buffer, 5x stock gel regenerant and a ready to use IgG purification gel. Briefly, the pools were dialysed against purification buffer for 2 hours at ambient temperature with replacement of the buffer after one hour. A funnel with filter paper leading into a filter flask, which was attached to a vacuum supply was used to drain buffer from the gel. Equal volumes of gel and culture medium pool were mixed in a 50 ml tube and incubated for 5 minutes on a nutating mixer (Karl Hecht, Sondheim, Germany). The slurry was applied to a new filter and the purified sample collected in a clean flask by applying vacuum. Between the different medium pools, the gel was regenerated using gel regenerant, followed by extensive washing with 10 volumes of purification buffer. The filtrates were centrifuged for 5 minutes at 2100 x*g* to remove some remaining gel particles. The immunoglobulins were precipitated from the filtrates by dropwise addition of an equal volume (ranging from 12.5 to 22.0 ml) of saturated ammonium sulphate solution (Pierce). After incubation for 30 minutes at room temperature, the proteins were pelleted by centrifugation during 20 minutes at 2100 x*g* at room temperature. The pellets were washed once with 1 ml 1:1 diluted saturated ammonium sulphate solution and dissolved in 0.5 ml of ultrapure water. The protein solutions were dialysed at room temperature against 500 ml of PBS using dialysis tubing with a 12/14,000 Dalton cutoff (Spectra/Por, Breda, the Netherlands), followed by exchange of PBS and another dialysation step overnight at 4 °C.

The immunoglobulins were conjugated with HRPO using a method based on periodate activation (Wilson and Nakane, 1976). Briefly, 20 mg of EIA-grade HRPO (Roche, Almere, the Netherlands) was dissolved in 5 ml of sterile ultrapure water. After addition of 1 ml of a 0.1 M sodium periodate solution in 20 mM acetate-buffer pH 4.4, the solution was incubated for 20 minutes at room temperature and subsequently dialysed overnight against 1 mM acetate buffer pH 4.4 at 4 °C. The immunoglobulin content of the filtrates was determined by A280 measurement. The

pH of the filtrates was adjusted to 9.5 by addition of 0.36 ml of 0.2 M carbonate buffer. The pH of the HRPO-dialysate was adjusted accordingly, after which the HRPO-solution was immediately added to the IgG solutions (in a 2:1 w/w IgG:HRPO ratio). The suspensions were incubated for 2 h at room temperature with periodical gentle mixing. To each tube, 50 µl of a freshly prepared 4 mg/ml sodium borohydride solution in ultrapure water was added and mixed. The solutions were then incubated for 2 h at 4 °C with periodical gentle mixing. The conjugates were then precipitated by dropwise addition of an equal volume of saturated ammonium sulphate solution. After incubation for 15 minutes at room temperature, the proteins were pelleted by centrifugation for 10 minutes at 2100 x*g*. The pellets were dissolved in 0.5 ml of ultrapure sterile water and dialysed against 500 ml of PBS for 1 h at room temperature. After exchange of PBS dialysation was continued overnight at 4 °C.

To the conjugate dialysates a 1.5x volume of 87% glycerol (Merck) was added, and after gentle thorough mixing by pipette, the conjugates were aliquoted in 0.5 ml portions and stored at -20 °C.

The conjugates were tested in a two-step direct ELISA using crude and detergent extracted *H. meleagridis* and control antigens to select a MAb-conjugate reactive with histomonal antigens and a low background when tested on control antigens. One MAb-conjugate (1B10B11) was selected for use in the blocking-ELISA.

Blocking-ELISA. A blocking-ELISA was developed by performing checkerboard titrations of crude *H. meleagridis* antigen, MAb-conjugate 1B10B11, and positive and negative sera, followed by optimization of the test protocol: crude *H. meleagridis* antigen was diluted 1/2400 in PBS and 100 µl added to a high-binding ELISA-plate. After coating overnight at room temperature, the plates were emptied and blocked with 200 µl/well of 1% BSA in PBS for 45 minutes at room temperature. The plates were washed three times with ultrapure water with 0.05% tween 80. Sera were diluted 1/5 in ELISA-buffer, consisting of PBS with 0.1 % BSA, 0.05% tween 80 and 2% extra NaCl and 100 µl was added per well. After an incubation of 1 h at room temperature, the plates were washed three times again and 100 µl of the MAb-conjugate diluted 1/2000 in ELISA buffer, was added to each well. After another incubation of 1 h at room temperature, the plates were washed five times. Then, 100 µl of substrate solution (see indirect ELISA) was added per well. The plates were incubated for 15 minutes at room temperature. The enzyme reaction was stopped by the addition of 50 µl 1 M sulphuric acid. OD's were measured at a wavelength of 450 nm. For each sample a percentage of inhibition (PI) of the sample OD in comparison with the average OD obtained for buffer instead of sample, was calculated. The cut-off was set at a PI of 50%.

The repeatability of the blocking-ELISA was determined by including a negative and a positive reference sample in every test. The reproducibility was

checked by testing the same set of samples (n=324) in two assays performed on different test dates by two different technicians, using a different batch of *H. meleagridis* antigen.

***H. meleagridis* sera**. In order to optimize an animal model for *in vivo* testing of antihistomonals (Van der Heijden and Landman, 2008a) experimental inoculations were performed in chicken and turkey poults. During these experiments also sequential blood samples were collected for development and validation of the blocking-ELISA. Briefly, nine two week old BUT6 turkeys poults and ten Isa Brown chicks, kept in isolators, were intracloacally inoculated with 1 ml of a viable *H. meleagridis* culture as described in detail elsewhere (Van der Heijden and Landman, 2008a). At the termination of the experiment the birds were transported to the postmortem room, stunned with carbon dioxide and bled. After clotting, serum was collected by centrifugation and stored at -20 °C until used.

Tetratrichomonas gallinarum. *T. gallinarum* strain M3 (Cepicka et al., 2005) was cultured in Diamonds TYM medium (Diamond, 1957) supplemented with 10% horse serum and 0.5% agar (Oxoid, Basingstoke, United Kingdom) at 40 °C after resuscitation from liquid nitrogen. After one day the culture was subcultured in 5 tubes, each by inoculating 1 ml of *T. gallinarum* culture into 9 ml of TYM medium. The cultures were incubated for one day at 40 °C and subsequently pooled. After centrifugation for 10 minutes at 1000 x*g* at 20 °C a pellet consisting of agar and parasites was obtained. Fourfold repeated melting of the agar at 40 °C, addition of prewarmed PBS, resuspension of parasites, and centrifugation, yielded a pellet that only contained protozoa. The pellet was suspended in 1 ml of PBS. From this suspension, which contained approximately $1.2x10^8$ protozoa/ml, a crude *T. gallinarum* antigen was derived by a freeze-thaw step. The crude antigen was stored at -20 °C until used.

An indirect ELISA for *T. gallinarum* similar to the indirect *H. meleagridis* ELISA was developed, using the *T. gallinarum* crude antigen as coating antigen at a 1/4000 dilution.

Antisera against *T. gallinarum* were raised in SPF layers. Three groups of 18 chicken of 16 weeks of age, were kept in isolators (1.94 m x 0.74 m x 0.95 m, Beyer & Eppelaar, the Netherlands). After one week, the first group (negative controls) were intracloacally inoculated with 1 ml of TYM medium. The second group was intracloacally inoculated with 1 ml of a culture of *T. gallinarum* diluted with TYM medium to a concentration of 20 000 protozoa/ml (Kemp and Reid, 1965). The third group was intramuscularly immunized with *T. gallinarum* antigen, diluted 1/100 in PBS and emulsified with Freunds incomplete adjuvant. Blood samples were collected at -1, 2, 4 and 5 weeks after inoculation or immunization.

The sera were tested in the indirect *T. gallinarum* ELISA and in the *H. meleagridis* blocking-ELISA.

Results

Three of the five MAb-producing hybridoma clones had high OD's (around 2.0) in the indirect *H. meleagridis* ELISA. No decline of OD was seen upon multiple passaging, indicating a stable antibody production with time.

At immunohistochemistry all three MAb's detected histomonads in liver tissue of a *H. meleagridis* infected turkey. Besides the clearly (brown) stained histomonads sometimes slight staining of surrounding tissue was observed (Figure 1).

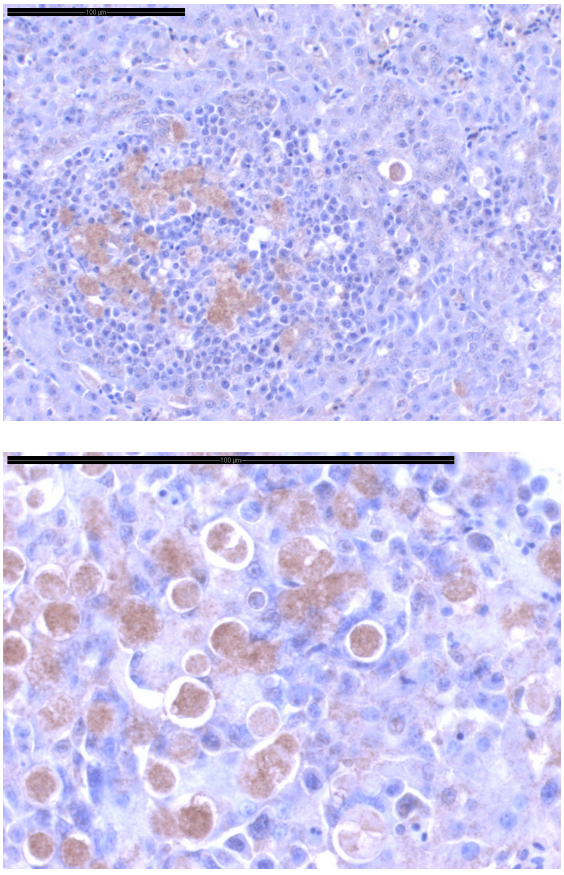


Figure 1: Liver tissue sections originating from a turkey infected with *H. meleagridis* shown at two different magnifications. Immunochemistry using monoclonal antibody 1B10B11. Bars = 100 µm.

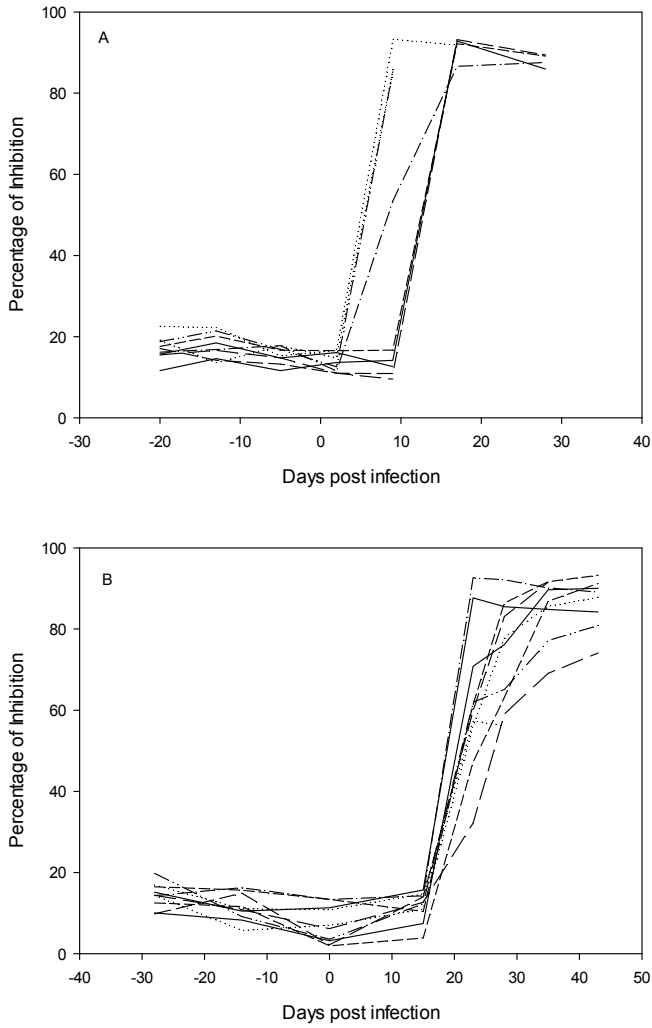


Figure 2: Analysis of experimental sera in the *H. meleagridis* blocking-ELISA.
A: Turkeys. B: Chicken.

After conjugation, MAb 1B10B11 was found to be reactive against both detergent extracted and crude *H. meleagridis* antigens in a two-step direct ELISA, while the OD's obtained using detergent extracted and crude control antigens were very low (background level). Also background level OD's were obtained when the

MAb conjugate was tested in a two-step direct ELISA using *T. gallinarum* antigen. When the conjugate was titrated against *H. meleagridis* antigens, a 1/2000 dilution resulted in an OD of approximately 1.0. This dilution was chosen as the working dilution for the blocking-ELISA.

For both chicken and turkey, experimental sera were tested serially diluted in a blocking-ELISA's using either detergent extracted and crude *H. meleagridis* antigens. The results on both type of antigens were almost identical, and crude antigen was selected for further testing. Pre-infection sera had low PI's at each dilution tested, while titres of end sera ranged between 20 and 160. A dilution of 1/5 was selected as an appropriate sample dilution for the blocking-ELISA.

The repeatability of the *H. meleagridis* blocking-ELISA was high, since in two test runs of 9 ELISA plates, the negative sample had an average PI of 17.5 with a standard deviation of 3.0, while the positive sample gave an average PI of 88.6 with a standard deviation of 1.0. The test also had a high reproducibility. There was an excellent agreement of 98.4% of the qualitative results of the two test runs (324 samples), with also a high correlation between the PI of the samples in both tests ($r = 0.96$; slope 1.02).

Except for two birds that died from histomonosis before a humoral response could develop, for all experimentally inoculated turkeys a clear rise in PI was obtained following inoculation (Figure 2A). Four turkeys already had high PIs at 9 dpi, while at the next sampling at 17 dpi all remaining birds showed a high PI. Eight of ten experimentally inoculated chickens (Figure 2B) seroconverted between 15 and 23 dpi, while two birds had PIs just below the cut-off level, but were seropositive at the next sampling at 28 dpi.

The chickens immunized with the *T. gallinarum* antigen showed a clear increase in OD in the indirect *T. gallinarum* ELISA, while for the groups of birds intracloacally inoculated with either *T. gallinarum* or a sham inoculum no increase in OD was found. When these samples were tested in the *H. meleagridis* blocking-ELISA the increase in PI in time was small and no significant difference (Students t-test, $P<0.05$) between the three groups (pairwise comparisons) was observed on 2, 4, or 5 weeks post inoculation.

Discussion

H. meleagridis is antigenically related to *T. gallinarum*, an intestinal flagellate which is common in poultry and other birds (Cepicka et al., 2005). Hyperimmune antisera raised in rabbits against the two flagellates cross-reacted in an indirect fluorescent antibody test (Dwyer, 1972a) although in agar gel immuno diffusion test (Dwyer, 1972b) species-specific precipitin lines were seen. Both antigenic differences and some cross-reactivity were also seen in immunoelectrophoresis (Dwyer, 1974).

To ascertain a specific test for *H. meleagridis*, MAbs were developed against a detergent extracted protein. Triton-X-114 extraction and subsequent phase separation is used for isolation of cell membrane associated proteins (Hemphill and Gottstein, 1996). Therefore, probably, the MAb's are directed against membrane proteins of *H. meleagridis*.

When the MAb's were applied for immunohistochemistry, histomonads were readily detected in liver sections originating from *H. meleagridis* infected turkeys, confirming the fact that the MAbs were directed against *H. meleagridis.* During screening of the hybridoma's an indirect ELISA was used with the selection criterion that a high OD should be found for *H. meleagridis* antigen in combination with a low OD for control antigen, which was prepared from a culture containing all ingredients of a *H. meleagridis* culture (medium, horse serum, rice powder and bacterial flora) except *H. meleagridis* itself.

After conjugation, the MAb reacted with both detergent-extracted and crude antigens of *H. meleagridis*, but not with control antigens, and also not with *T. gallinarum* antigen. It was therefore unlikely that *T. gallinarum* antibody positive birds would test positive in the blocking-ELISA employing this MAb. This was assessed by producing *T. gallinarum* antisera in chicken. Since *T. gallinarum* is considered non-pathogenic and non-invasive, we anticipated on finding that no serological response would occur in intracloacally inoculated birds. Therefore, a group of birds was intramuscularly injected with *T. gallinarum* antigen to ensure a humoral response against this organism. As expected only these birds seroconverted in the indirect *T. gallinarum* ELISA. The negative control group and the group of chickens which were intracloacally inoculated showed no increase in OD in this ELISA following inoculation. The *T. gallinarum* antibody positive group of birds showed low PI's in the *H. meleagridis* blocking-ELISA. Therefore, it was concluded that the *H. meleagridis* blocking-ELISA does not suffer from cross-reactivity against the closely related flagellate *T. gallinarum*.

During the last 5 years, histomonosis research following its re-emergence has yielded a number of new laboratory and diagnostic tools, to which the blocking-ELISA presented here, can be added. The test may prove useful for diagnostic and research purposes. Candidate products with possible antihistomonal activity can be screened using *in vitro* (Callait et al, 2003; Zenner et al, 2003; Grabensteiner et al, 2007; Hauck & Hafez, 2007; Van der Heijden and Landman, 2008a) and *in vivo* models (Hu and McDougald, 2004; Hafez and Hauck, 2006; Van der Heijden and Landman, 2008b). In the *in vivo* models, usually all birds are inoculated and the antihistomonal effect of a candidate product is estimated using the degree of morbidity and mortality in the experimental group in comparison with the untreated control group. As it has been reported previously that *H. meleagridis* can spread quickly within a turkey flock by direct lateral transmission (Hu and McDougald, 2003), a transmission model where seeder birds are used, probably mimics more closely a natural infection and could probably enable detection of a more subtle

antihistomonal effect of some compounds that remains undetected in standard *in vivo* models. In addition to morbidity and mortality, a serological tool like the blocking-ELISA in this study could provide more data about differences in transmission rates between groups.

Another interesting application of the blocking-ELISA could be seroepidemiological studies on chicken farms. In chickens, histomonosis is generally less severe and they form a reservoir of the parasite. However, it is not known what proportion of chicken flocks is infected, whether there are differences between housing systems, and if the disease is of economic relevance. The blocking-ELISA could be a cost and labour effective tool to perform a seroepidemiological study in chicken flocks in order to answer some of these questions.

Finally, the ELISA might contribute as a diagnostic tool in outbreaks of histomonosis in turkey flocks.

Acknowledgements

We thank Dr. J. Tachezy, Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic for kindly supplying *T. gallinarum* strain M3.

References

Augustine, P.C., Lund, E.E., 1968, Indirect fluorescent antibody test on *Histomonas*. Journal of Protozoology S 15, 19.

Augustine, P.C., Lund, E.E., 1970, Indirect fluorescent antibody tests comparing two strains of *Histomonas meleagridis* and *Histomonas wenrichi*. Journal of Protozoology 17, 97-99.

Callait, M.P., Granier, C., Chauve, C., Zenner, L., 2003, *In vitro* activity of therapeutic drugs against *Histomonas meleagrids* (Smith, 1895). Poultry Science 81, 1122-1127.

Cepicka, I., Kutisova, K., Tachezy, J., Kulda, J., Flegr, J., 2005, Cryptic species within the *Tetratrichomonas gallinarum* species complex revealed by molecular polymorphism. Veterinary Parasitology 128, 11-21.

Clarkson, M.J., 1963, Immunological responses to *Histomonas meleagridis* in turkey and fowl. Immunology 6, 156-168.

Diamond, L.S., 1957, The establishment of various trichomonads of animals and man in axenic cultures. Journal of Parasitology 43, 488-490.

Dwyer, D.M., 1970, An improved method for cultivating *Histomonas meleagridis*. Journal of Parasitology 56, 191-192.

Dwyer, D.M., 1971, Immunologic analysis by gel diffusion of effects of prolonged cultivation on *Histomonas meleagridis* (Smith). Journal of Protozoology 18, 372-377.

Dwyer, D.M., 1972a, Analysis of antigenic relationships among *Trichomonas*, *Histomonas*, *Dientamoeba*, and *Entamoeba*. I. Quantitative fluorescent-antibody methods. Journal of Protozoology 19, 316-325.

Dwyer, D.M., 1972b, Analysis of antigenic relationships among *Trichomonas*, *Histomonas*, *Dientamoeba*, and *Entamoeba*. II. Gel-diffusion methods. Journal of Protozoology 19, 326-332.

Dwyer, D.M., 1974, Analysis of antigenic relationships among *Trichomonas*, *Histomonas*, *Dientamoeba* and *Entamoeba*. III. Immunoelectrophoresis techniques. Journal of Protozoology 21, 139-145.

Dwyer, D.M., Honigberg, B.M., 1972, Immunological analysis by quantitative fluorescent-antibody methods of effects of prolonged cultivation on *Histomonas meleagridis* (Smith). Zeitschrift für Parasitenkunde 39, 39-52.

Grabensteiner, E., Arshad, N., Hess, M., 2007, Differences in the *in vitro* susceptibility of mono-eukaryotic cultures of *Histomonas meleagridis*, *Tetratrichomonas gallinarum* and *Blastocystis* sp. to natural organic compounds. Parasitology Research 101, 193-199.

Hafez, M.H., Hauck, R., 2006, Efficacy of an herbal product against *Histomonas meleagridis* after experimental infection of turkey poults. Archives of Animal Nutrition 50, 1-7.

Hall, C.F., Krishnam, P.V., 1967, The demonstration of *Histomonas Meleagridis* in tissues and cell cultures using fluorescent labeled antibody techniques. Journal of the American Veterinary Medical Association 150, 1339-1339.

Hauck, R., Hafez, M.H., 2007, Effect of coated plant extracts on *Histomonas meleagridis* and growth of bacteria *in vitro*. Avian Diseases 51, 880-883.

Hemphill, A., Gottstein, B., 1996, Identification of a major surface protein on *Neospora caninum* tachyzoites. Parasitology Research 82, 497-504.

Hu, J.H., McDougald, L.R., 2003, Direct lateral transmission of *Histomonas meleagridis* in turkeys. Avian Diseases 47, 489-492.

Hu, J.H., McDougald, L.R., 2004, The efficacy of some drugs with known antiprotozoal activity against *Histomonas meleagridis* in chickens. Veterinary Parasitology 121, 233-238.

Kemp, R.L., Reid, W.M., 1965, Pathogenicity studies on *Trichomonas gallinarum* in domestic poultry. Poultry Science 44, 215-221.

McDougald, L.R., 1998, Intestinal protozoa important to poultry. Poultry Science 77, 1156-1158.

McDougald, L.R., 2005, Blackhead disease (histomoniasis) in poultry: a critical review. Avian Diseases 49, 462-476.

Singh, A., Weissenbock, H., Hess, M., 2008, *Histomonas meleagridis*: Immunohistochemical localization of parasitic cells in formalin-fixed, paraffin-embedded tissue sections of experimentally infected turkeys demonstrates the wide spread of the parasite in its host. Experimental Parasitology 118, 505-513.

Van der Heijden, H.M., Landman, W.J., 2007, Improved culture of *Histomonas meleagridis* in a modification of Dwyer medium. Avian Diseases 51, 986-988.

Van der Heijden, H.M., Landman, W.J., 2008a, *In vitro* effect of herbal products against *Histomonas meleagridis*. Veterinary Parasitology 154, 1-7.

Van der Heijden, H.M., Landman, W.J., 2008b, *In vivo* effect of herbal products against *Histomonas meleagridis* in turkeys. Avian Pathology 37, 45-50.

Van der Heijden, H.M., Landman, W.J., Greve, S., Peek, R., 2006, Genotyping of *Histomonas meleagridis* isolates based on internal transcribed spacer-1 sequences. Avian Pathology 35, 330-334.

Wessel, D., Flugge, U.I., 1984, A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Analytical Biochemistry 138, 141-143.

Wilson, M.B., Nakane, P.K., 1976, The covalent coupling of proteins to periodate-oxidized sephadex: a new approach to immunoadsorbent preparation. Journal of Immunological Methods 12, 171-181.

Zenner, L., Callait, M.P., Granier, C., Chauve, C., 2003, *In vitro* effect of essential oils from *Cinnamomum aromaticum*, *Citrus limon* and *Allium sativum* on two intestinal flagellates of poultry, *Tetratrichomonas gallinarum* and *Histomonas meleagridis*. Parasite 10, 153-157.

Chapter 5

Molecular typing and phylogeny

Chapter 5.1

Genotyping of *Histomonas meleagridis* isolates based on internal transcribed spacer-1 sequences

H.M.J.F. van der Heijden[1], W.J.M. Landman[1], S. Greve[2] and R. Peek[2]

1. Animal Health Service Ltd, P.O. Box 9, 7400 AA Deventer, the Netherlands
2. University of Amsterdam, Academic Medical Center, Department of Microbiology, Section Parasitology, Meibergdreef 9, 1105 AZ Amsterdam, the Netherlands

Published: Avian Pathology 35 (2006) 330-334

Abstract

C-profiling is a novel genotyping method for protozoan pathogens, based on polymerase chain reaction and sequencing of AT-rich Internal Transcribed Spacer-1 sequences. It was applied to various *Histomonas meleagridis* isolates originating from outbreaks of histomonosis in six Dutch turkey and chicken flocks. Three different *H. meleagridis* genotypes were identified. Type I and type II were associated with clinical disease. In two flocks, both having recovered from an outbreak of histomonosis, a type III strain was found that was also morphologically slightly different from the type I and type II isolates. C-profiling is a promising technique to differentiate between *H. meleagridis* subtypes, making it useful for epidemiological studies.

Introduction

The flagellated protozoan *Histomonas meleagridis* (Tyzzer and Fabyan, 1920) is the cause of histomonosis or blackhead disease in gallinaceous fowl (Smith, 1895). The disease is re-emerging in the European community after effective chemotherapeutics including arsenicals and nitroimidazoles were banned following increasing concern about drug residues in food and the identification of some compounds as potentially carcinogenic (Zenner et al., 2003). In turkeys Histomonas-induced mortality is high, whereas in chickens disease is generally less severe (McDougald, 1997). Chicken flocks may form a reservoir for *H. meleagridis* and readily infect turkeys by either direct contact or through infected litter with eggs from *Heterakis gallinarum* acting as a vector (Graybill and Smith, 1920; Tyzzer, 1934). The possibility of wild birds as a reservoir has not been ruled out. The disease has been described in a number of species, including pheasants (Lund and Chute, 1972a,b; Wernery and Kinne, 2002), quail, and partridges (Lund and Chute, 1972b).

Although differences in pathogenicity of isolates have been observed (Dwyer and Honigberg, 1970), so far no subtyping of *Histomonas* isolates has been reported. Identification of subtypes might however prove useful in epidemiological studies, evaluation of correlation of infections between flocks or species, for monitoring the efficiency of treatment, assessment of differences in virulence between strains, and so on.

H. meleagridis is a trichomonad, morphologically (Johnson et al., 2004) and antigenically (Dwyer 1972a,b, 1974) closely related to *Dientamoeba fragilis*, a non-flagellated protozoan pathogenic to humans. Also the phylogenetic relation of these two trichomonads, based on sequence analysis of small subunit rRNA genes, is very close (Gerbod et al., 2001). The small subunit rRNA gene, and especially the ribosomal gene region containing the Internal Transcribed Spacer-1 (ITS-1)/5.8S/ITS-2, has been used for extensive phylogenetic analysis of Trichomonadidae (Felleisen, 1997; Gerbod et al., 2001; Cepicka et al., 2005; Kleina et al., 2004; Kutisova et al., 2005). Its usefulness for genotyping *H. meleagridis* has not yet been assessed. In the present study, ITS-1 sequences of *H. meleagridis* isolates are compared using a novel technique that we developed: C-profiling. This method is based on the fact that, due to multiple ITS-1 sequence variants in a single isolate, sequencing fluorograms are too complex for direct interpretation. But since the C-content is rather low, more simple patterns are produced by deleting in the fluorogram the peaks representing the other nucleotides (A, T and G). A single C nucleotide is then represented by multiple peaks of different heights due to small differences in size of the individual ITS-1 variants due to insertions, deletions and mutations involving C, and by the relative proportions of the different ITS-1 variants present in the polymerase chain reaction (PCR) product of an isolate.

Materials and Methods

***H. meleagridis* isolates**. An isolation procedure for *H. meleagridis* as described in more detail elsewhere (Van der Heijden et al., 2005) was applied to birds from several sources. Briefly, immediately after euthanasia, caeca were opened and caecal fluid or content was inoculated into Dwyer's medium and incubated at 40 °C. After 48 h, cultures that contained histomonads were subcultured at least once (up to 23 passages). Samples of freshly inoculated medium or (sub)cultures (with or without visible histomonads) were stored in liquid nitrogen by adding 10% dimethylsulphoxide (Merck, Germany) as a cryoprotectant, aliquoting into vials of 1 ml and freezing to -70 °C under controlled conditions (approximately -1 °C per min) before transfer to liquid nitrogen (-175 °C).

Birds examined. Eight turkeys with liver and caecal lesions originating from a flock (K) with acute histomonosis were cultured for *Histomonas*, and *H. meleagridis* was isolated from them all. Six birds with moderate to severe liver and caecal lesions were necropsied from a chicken flock (B) with acute histomonosis, and *H. meleagridis* was successfully isolated and subcultured from two. An outbreak of clinical histomonosis occurred in turkeys on a farm (S) with both turkeys and chickens, and a turkey (St) and a chicken (Sc) isolate of *H. meleagridis* were obtained. From the same flock (St') 2 months after the initial outbreak of disease, but now with no clinical histomonosis, isolation of *H. meleagridis* was attempted from turkeys in the group that survived the disease. Culture was also attempted from four birds in a turkey flock (H) 1 month after an unconfirmed outbreak and from 16 birds in a chicken flock (G) with an outbreak of 6-week duration. Finally, caecal contents from two SPF turkeys were inoculated into medium to serve as negative controls.

Polymerase chain reaction. The ITS-1 region of *H. meleagridis* was amplified by PCR using a forward primer ssu2 (5'-ggaatcccttgtaaatgcgt-3') based on ssu rRNA genes of *H. meleagridis* (AF293056), *D. fragilis* (U37461) and *T. vaginalis* (AY677680), and a reverse primer 5.8s1 (5'-tgtgaggagccaagacatcc-3') complementary to the 5' end of the 5.8S ribosomal RNA gene of *H. meleagridis* and *D. fragilis*. The 5.8S rRNA gene sequence of these organisms as a basis for the design of the reverse primer was determined by PCR amplification of the ITS-1/5.8S/ITS-2 region using forward primer ssu2 and a reverse primer lsu1 (5'-agttcagcgggtcttcctg-3'), complementary to a conserved region in the 5' end of the large subunit RNA genes of Trichomonad species, followed by cloning and sequencing.

For PCR, DNA was isolated from 100 µl *H. meleagridis* culture (fresh or after storage in liquid nitrogen) by diluting the culture 1:2 with phosphate-buffered saline

and using a commercial DNA isolation kit (Qiamp DNA Minikit, Qiagen) according to the manufacturer's instructions.

PCR was performed using a reaction volume of 50 µl consisting of 30% DNA template, 0.3 µM each primer, 500 µM dNTPs, 10% of 10x PCR buffer (Promega), 3 mM $MgCl_2$, and 0.1 mg/ml bovine serum albumin, and 0.4 mg/ml α-casein to eliminate PCR inhibitors, and 1 U Taq polymerase (Promega). A PCR run consisted of 40 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec, and elongation at 72 °C for 30 sec. Amplification products were size fractionated on 1% agarose gels (80 V, 30 min) and visualized by ethidium bromide staining.

Cloning and sequencing. One microlitre of PCR product of a number of *H. meleagridis* isolates was ligated in the PCR cloning pGEMTeasy vector (Promega) as described by the manufacturer. After transformation into Escherichia coli DH5α, individual clones were tested in PCR using either the ssu2/lsu1 (ITS-1/5.8S/ITS-2 region) or the ssu2/5.8s1 primers (ITS-1 region). Positive clones were sequenced using an Applied Biosystems Prism 310 dye terminator-fluorescence based genetic analyser. Initially only the ssu2 primer was used for sequencing. To confirm the results of ITS-1 sequencing, PCR was repeated and sequencing performed using the 5.8s1 primer. Homology searches were performed using the Blast program with default settings (http://www.ncbi.nlm.nih.gov/blast/). Multiple sequence alignments and C-profiles were produced using the CodonCode software (CodonCode Corporation, Dedham, USA).

Genbank submission. The 3' part of the ssu rRNA and ITS-1 region of the nine different clones of *H. meleagridis* type III was deposited in GenBank with accession numbers DQ350439 to DQ350447.

Results

ITS-1 sequence of *H. meleagridis*. The PCR amplified the 3' end of the ssu rRNA gene and the complete ITS-1 region of *H. meleagridis* DNA isolated from culture. The amplicon was approximately 300 base pair (bp) in size. Sequence analysis of PCR products from several *H. meleagridis* isolates resulted in ITS-1 sequences with many ambiguities in sequencing fluorograms (data not shown), indicating a heterogeneous ITS-1 sequence within a single isolate or trophozoite. Using the same primers a similarly variable ITS-1 sequence with many ambiguities was also observed in *D. fragilis* but not in *T. vaginalis* (data not shown).

C-profiling. In total 80 *H. meleagridis* isolates were tested by PCR, resulting in 68 positive results - of which 46 PCR products were C-profiled. This resulted in three different C-profiles (Figure 1).

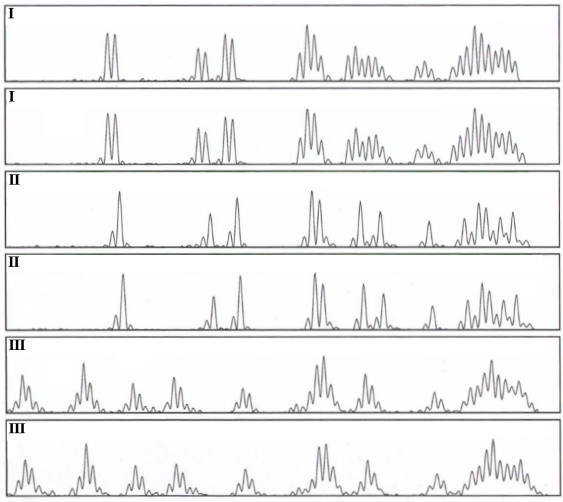


Figure 1. C-profiling of *H. meleagridis* ITS-1 and 5.8 S region: examples of C-profiles of two isolates per type I, II or III.

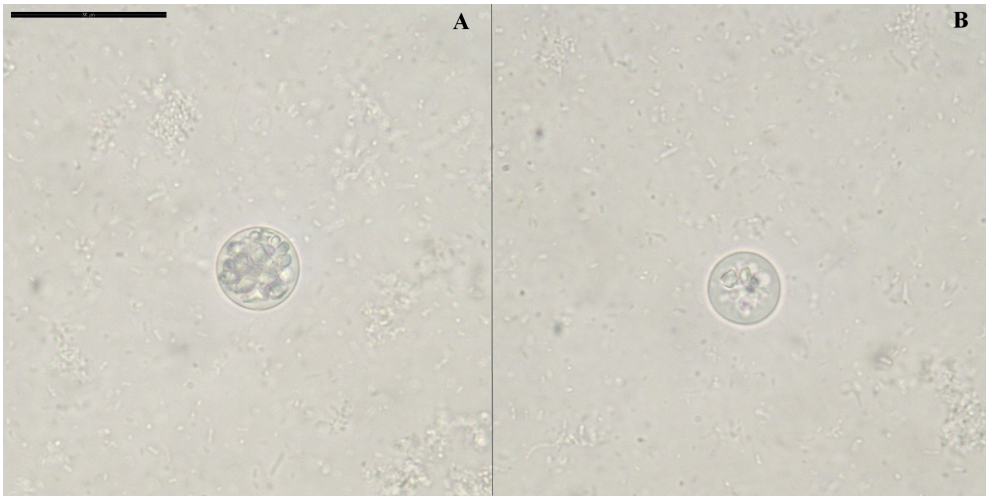


Figure 2. Dixenic cultures: 2a: a unicellular organism with slightly different morphology. 2b: *Histomonas meleagridis*. Bar = 30 µm.

Type I and type II profiles were more alike than the type III profile. This type III was found in three unrelated isolates from both chickens and turkeys. At the time of isolation it was observed upon microscopic examination of these cultures that these unicellular organisms had a morphology that was distinct from *H. meleagridis*; that is, they were slightly larger in size, with a more granular appearance and no visible flagellum (Figure 2). Like *H. meleagridis*, this organism also ingested rice particles. However, it was not possible to subculture these protozoa in Dwyer's medium or to re-start culture from cryopreserved isolates stored in liquid nitrogen. Phylogenetic analysis of the sequence of the 150 bp at the 3' end of ssu rRNA of the type III strains showed that this organism is closely related to *H. meleagridis* and *D. fragilis* and less related to other trichomonads.

C-profiling proved highly reproducible and gave consistent results for a particular sample or isolate (data not shown). Also, subculturing of type I or type II strains (the organism with a type III C-profile could not be subcultured) did not result in changes in the C-profile.

For each of the three C-profiles, the DNA products of an isolate were cloned to analyse the sequence variability within the ITS-1 region. The ITS-1 region of nine or 10 individual clones per isolate was fully sequenced (Table 1) and proved extremely AT-rich (between 90 and 94%). The ITS-1 length of type I or type II clones was on average 78 bp, while ITS-1 of type III clones was on average 93 bp long. Some clones had identical sequences to others. For the 19 type I or type II clones in total 11 different sequences were found, while for the 10 type III clones nine different sequences were found. The type I clone sequences were similar to type II clone sequences with differences due to base substitutions and inserts or deletions of one or two bases. Initially, sequencing was done with a single primer (ssu2). To confirm the sequencing results, PCR was repeated followed by sequencing using reverse primer 5.8s1. A few clones proved repeatedly PCR-negative and could not be sequenced using primer 5.8s1.

A 10-fold limiting dilution from a type I and a type II strain of *H. meleagridis* was made. From each dilution step DNA was isolated and tested in PCR, and the positive PCR products were C-profiled. The C-profiles were consistent, indicating that the ITS-1 variation is intragenomic.

The 68 PCR-positive isolates came from various sources. For birds that were culture-negative, only primary samples were available (medium inoculated with caecal contents); whereas for culture-positive birds, in addition samples from various passages after subculturing were stored in liquid nitrogen. DNA was isolated either directly from these samples, or from culture media after successfully restarting culture. Within a flock (and in several samples from a single bird) we never observed a mixed infection with type I and type II strains (Table 2). Type I was detected in all eight birds of a single turkey flock (K) that suffered from an acute outbreak of histomonosis, while type II strains were found in three flocks (B, G, and S).

Table 1: Sequence alignment of the ITS-1 regions of 29 *H. meleagridis* type I, II, or III clones.

Type/clone	Sequence
	10 20 30 40 50 60 70 80 90
I/1	GTTTAGTAAATAAAATTTAAACAATAATTTTAAAAATAAAAAAATTGAATACAAAATTAATTTTCAATCATTAATTAAA--
I/3, I/4, I/8[a], I/10[a]	GTTTAGTAAATAAAATTTAAACAATAATTTTAAAAATAAAAAAATTAAATACAAAATTAATTTTCAATCATTAATTAAA--
I/5, I/7, II/1, II/2	GTTTAGTAAATAAAATTTAAACAATAATTTTAAAAATAAAAAAATAATATACAAAATTAATTTTCAATCATTAATTAAA--
I/9[a]	GTTTAGTAAATAAAATTTAAACAATAATTTTAAAAATAAAAAAATAATATACAAAATTAATTTTCAATCTTAAAATTTA--
I/6	GTTTAGTAAATAAAATTTAAACAATAATTTTAAAAATAAAAAA-TTAAATACAAAATTAATTTTCAATCATTAATTAAA--
I/2	GTTTAGTAAATAAAATTTAAACAATAATTTTAAAAATAAAAAAATTATATACAAAATTAATTTTCAATCATTAATTAAA--
II/6	GTTTAGTAAATAAAATCTAAACAATAATTTTAAAAATAAAAAAATTAAATACAAAATTAATTTTCAATCATTAATTAAA--
II/7	GTTTAGTAAATAATATTTAAACAATAATTTTAAAAATAAAAAA-TAATATACAAAATTAATTTTCAATCATTAATTAAA--
II/4, II/8[a], II/9[a]	GTTTCGTAAATAAAATTTAAACAATAATTTTAAAAATAAAAAAATAATATACAAAATTAATTTTCAATCATTAATTAAA--
II/3	GTTTAGTAAATAAAATTTAAACAATAATTTTAAAAATAAAAAAATTAAATACAAAATTAATTTTCAATCTTAAAATTTAAA
II/5	GTTTAGTAAATAAAATTTAAACAATAATTTTAAAAATAAAAAAATAATATACAAAATTAATTTTCAATCTTAAAATTTAAA
III/1	GTTTCGTTTTTTAAATAAAAAAAAAA-CAATAATTTTTTT---AAATTAAACGAAAATTTATACAAAATTCAAAATCAATAAATTTCAAAAATTCAA
III/2, III/4	GTTTAGTTTTTTATTTAAAAAAAAA--CAATAATTTTTTT-ATAAAATTTCA--CAATTTATACAAAATTCAAAATCATTAAATTTCAAAAATTCAA
III/3	GTTTAGTTTTTTATTTAAAAAAAA---CAATAATTTTTTTTATGAAATTTAA--CAATTTATACAAAATTCAAAATCAATAAATTTCAAAAATTCAA
III/5	GTTTAGTTTTTTATTTTAAAAAAAAA-CAATAATTTTTTT-ATAAAATTTAA--CAATTTATACAAAATTCAAAATCAATAAATTTCAAAAATTCAA
III/6	GTTTAGTTTTTTATTTAAAAAAAAAAC-AATAATTTTTTTTATAAAATTTAA--CAATTTATACAAAATTCAAAATCACTAAATTTCAAAAATTAA-
III/7	GTTTAGTTTTTTATTTAAAAAAAAA--CAATAATTTTTTTTATAAAATTTAA--CAATTTATACAAAATTCAAAATCAATAAATTTCAAAAATTCAA
III/8	GTTTCGTTTTTTATTTAAAAAAAAA--CAATAATTTTTTT--AAAATTAAATAAAAATTTATACAAAATTCAAAATCAATAAATTTCAAAAATTAAA
III/9	GTTTAGTTTTTTATTTAAAAAAAAAAACAATAATTTTTTTTATAAAATTTAG--CAATTTATACAAAATTCAAAATCAATAAATTTCAAAAATTAA-
III/10	GTTTAGTTTTTTATTTAAAAAAAAAAACAATAATTTTTTTTATAAAATTTAA--CAATTTATACAAAATTCAAAATCAATAAATTTCAAAAATTAAA

[a] Results based on sequencing using only primer ssu2.

Table 2: Results of C-profiling on *H. meleagridis* field strains from various sources.

Flock	Species	n	PCR+	Number of C-profiles	C-profiling		
					Type I	Type II	Type III
K	turkey	8	8	25	25	0	0
B	chicken	6	3	11	0	10	1
St	turkey	1	1	2	0	2	0
Sc	chicken	1	0	nd			
St'	turkey	1	1	2	0	0	2
H	turkey	4	1	1	0	0	1
G	turkey	16	8	5	0	5	0
SPF	turkey	2	0	nd			

n = number of bids examined in PCR, PCR+ = number of PCR-positive birds, # of C-profiles = total number of profiles produced.

These were all chicken flocks, although in flock S, with both turkey and chicken, the type II strain was isolated from the turkeys. Type III strains were isolated from single birds in three flocks (B, H and St'). Two of these flocks (H and St') had suffered from an outbreak of histomonosis several weeks before sampling. Specific pathogen free turkeys, serving as negative controls, gave negative PCR results.

Discussion

Although a PCR for specific detection of *H. meleagridis* based on amplification of part of the 18S rRNA gene has been described (Huber et al., 2005), so far no subtyping method for *H. meleagridis* had been published. Subtyping of *H. meleagridis* could be useful; for example, for epidemiological and pathogenesis studies, as well as for assessment of treatment efficiency and virulence differences between isolates. Variations in virulence between isolates have been examined with the aid of experimental inoculations, but it remained unclear whether these could be attributed to a strain effect (Dwyer and Honigberg, 1970). It is generally accepted that there are no differences between *Histomonas* strains from chickens and turkeys, and in the present study using C-profiling we found a type II *H. meleagridis* in both turkeys and chickens. Differences within the ITS regions have been frequently used as markers for subtyping in various organisms (Chilton and Gasser, 1999; Olivier et al., 2001) because these genes have no function in the mature ribosomes and therefore show more genetic variation than the coding regions. In the present work we describe a novel method, C-profiling, for subtyping of *H. meleagridis* based on DNA sequences of the ITS-1 region - rather than the ssu or lsu rRNA gene regions - that are very conserved among trichomonads. The

sequences of type I and type II clones were rather similar, but still clearly distinct C-profiles were obtained. Our assumption is that the relative proportions of different ITS-1 sequences in the genome of the *H. meleagridis* genotypes are probably very conserved.

The sequencing primer (ssu2) has a complete match with several *Tetratrichomonas gallinarum* strains (AY245111 to AY245121, AY245143 to AY245146, AY247746, AY247747 and AY247749), but partial sequences in the 18S region of the *H. meleagridis* isolates differed from sequences of corresponding regions in *T. gallinarum* (data not shown), and matched with *H. meleagridis* sequences (AF293056 or AJ920323). Therefore, it is unlikely that *T. gallinarum* interfered in our study.

Using C-profiling, consistent results were obtained for *H. meleagridis* strains - also after subculturing, storage below 0 °C, restarting and further subculturing. From these observations in combination with the limiting dilution experiments it was concluded that the ITS-1 variation observed in *H. meleagridis* was intragenomic (Wörheide et al., 2004) and not due to the presence of a mixture of *H. meleagridis* types in a single specimen.

In the limited number of outbreaks included in the present study, we were able to find three different C-profiles. Possibly more C-profiles would be identified in a larger survey including isolates from other countries. In clinical outbreaks, a type I or a type II strain was detected. In one case (flock G) a type II strain was also found 6 weeks after initial clinical disease. Culturing *H. meleagridis* from this flock proved difficult because of contamination of the cultures with an unidentified yeast (although *H. meleagridis* trophozoites were identified based on morphology). In three unrelated flocks a third C-profile (type III) was found. In the turkey flocks H and St' this type III strain was detected in birds from a group of turkeys that survived a clinical outbreak of histomonosis. This profile was also encountered in a single bird from chicken flock B suffering from an acute outbreak of histomonosis, while two other birds in this flock were infected with *H. meleagridis* type II. In all these cases isolation of *H. meleagridis* was attempted. Upon microscopic examination an amoeboid organism was seen that was morphologically similar to, but not identical to, *H. meleagridis*. It was slightly larger, with a more granular appearance, and no flagellum was visible. Although the organism, like *H. meleagridis*, ingested rice particles, it proved impossible to grow or subculture the organism in Dwyer's medium. Morphologically, the organism is different from *Tetratrichomonas gallinarum*, *Trichomonas gallinae*, or *Histomonas wenrichii* (Honigberg and Kuldova, 1969). Sequence analysis of the ssu rRNA gene revealed a close phylogenetic relationship between this organism and the human pathogen *D. fragilis* and with *Tritrichomonas foetus*, which is the common ancestor of *D. fragilis* and *H. meleagridis* (Gerbod et al., 2001) at a slightly larger distance. The sequence homology with *T. gallinarum* was less. Although it is well known that *D. fragilis* and *H. meleagridis* are closely related (Gerbod et al., 2001; Johnson et

al., 2004), it is remarkable that the ITS-1 sequence of this organism compares better with *D. fragilis* than with *H. meleagridis* type I and type II. It is hypothesized that this might be an amoeboid species in turkeys and chickens that has not been identified earlier because it is morphologically similar to (but not identical to) *H. meleagridis*. At present, it is not known whether this species also occurs in chicken or turkey flocks without a history of histomonosis or whether it occurs in other species of birds.

ITS-1 C-profiling can be considered a fingerprint technique for *H. meleagridis* for easy identification of substrains. The technique proved highly reproducible and easy to perform and interpret. At present only two (or three) different *H. meleagridis* C-profiles have been found, and for epidemiological studies a larger number of subtypes would be desirable. It would be useful if additional markers could be found in the ITS-2 part of the rRNA gene of *H. meleagridis*.

References

Cepicka, I., Kutisova, K., Tachzey, J., Kulda, J., Flegr, J., 2005, Cryptic species within the *Tetratrichomonas gallinarum* species complex revealed by molecular polymorphism. Veterinary Parasitology 128, 11-21.

Chilton, N.B., Gasser, R.B., 1999, Sequence differences in the internal transcribed spacers of DNA among four species of hookworm. International Journal of Parasitology 29, 1971-1977.

Dwyer, D.M., 1972a, Analysis of the antigenic relationships among *Trichimonas*, *Histomonas*, *Dientamoeba*, and *Entamoeba*. I. Quantitative fluorescent antibody methods. Journal of Protozoology 19, 316-325.

Dwyer, D.M., 1972b, Analysis of the antigenic relationships among *Trichimonas*, *Histomonas*, *Dientamoeba*, and *Entamoeba*. II. Gel diffusion methods. Journal of Protozoology 19, 326-332.

Dwyer, D.M., 1974, Analysis of the antigenic relationships among *Trichimonas*, *Histomonas*, *Dientamoeba*, and *Entamoeba*. III. Immunoelectrophoresis techniques. Journal of Protozoology 21, 139-145.

Dwyer, D.M., Honigberg, B.M, 1970, Effect of certain laboratory procedures on the virulence of *Histomonas meleagridis* for turkeys and chickens. Journal of Parasitology 56, 694-700.

Felleisen, R.S., 1997, Comparative genetic analysis of tritrichomonad protozoa by the random amplified polymorphic DNA technique. Parasitology Research 84, 153-156.

Gerbod, D., Edgcomb, V.P., Noel, C., Zenner, L., Wintjens, R., Delgado-Viscogliosi, P., Holder, M.E., Sogin, M.I. Viscogliosi, E., 2001, Phylogenetic position of the Trichomonad parasite of turkeys, *Histomonas meleagridis* (Smith) Tyzzer, inferred from small subunit rRNA sequence. Journal of Eukaryotic Microbiology 48, 498-504.

Graybill, H.W., Smith, T., 1920, Production of fatal blackhead in turkeys by feeding embryonated eggs of *Heterakis papillosa*. Journal of Experimental Medicine 31, 647-655.

Honigberg, B.M., Kuldova, J., 1969, Structure of a nonpathogenic histomonad from the cecum of galliform birds and revision of the Trichomonad family Monocercomonadidae Kirby. Journal of Protozoology 16, 526-535.

Huber, K., Chauve, C., Zenner, L., 2005, Detection of *Histomonas meleagridis* in turkeys cecal droppings by PCR amplification of the small subunit ribosomal DNA sequence. Veterinary Parasitology, 131, 311-316.

Johnson, E.H., Windsor, J.J., Clark, C.G., 2004, Emerging from obscurity: biological, clinical and diagnostic aspects of *Dientamoeba fragilis*. Clinical Microbiology Reviews 17, 553-570.

Kleina, P., Bettim-Bandinelli, J., Bonatto, S.L., Benchimol, M., Bogo, M.R., 2004, Molecular phylogeny of Trichomonadidae family inferred from ITS-1, 5.8S rRNA and ITS-2 sequences. International Journal of Parasitology 34, 963-970.

Kutisova, K., Kulda, J., Cepicka, I., Flegr, J., Koudela, B., Teras, J., Tachezy, J., 2005. Tetratrichomonads from the oral cavity and respiratory tract of humans. Parasitology 131, 309-319.

Lund, E.E., Chute, A.M., 1972a, The ring-necked pheasant (*Phasianus colchicus* torquatus) as a host for *Heterakis gallinarum* and *Histomonas meleagridis*. The American Midland Naturalist 87, 1-7.

Lund, E.E., Chute, A.M., 1972b, Reciprocal responses of eight species of galliform birds and three parasites: *Heterakis gallinarum*, *Histomonas meleagridis* and *Parahistomonas wenrichi*. The Journal of Parasitology 58, 940-945.

McDougald, L.R., 1997, Protozoa: other protozoan diseases of the intestinal tract. In: Calneck, B., Barnes, H.J., Beard, C.W., McDougald, L.R., Saif, Y.M. (Eds.), Diseases of Poultry. 10th ed. Iowa State University Press, Ames, pp. 890-899.

Olivier, C., Van de Pas, S., Lepp, P.W., Yoder, K., Relman, D.A., 2001, Sequence variability in the first internal transcribed spacer region within and among *Cyclospora* species in consistent with polyparasitism. International Journal of Parasitology 31, 1475-1487.

Smith, T., 1895, An infectious disease among turkeys caused by protozoa (infectious entero-hepatitis). US Department of Agriculture Bulletin 8, 7-90.

Tyzzer, E., 1934, Studies on histomoniasis, or ‘‘blackhead’’ infection, in the chicken and the turkey. Proceedings of the American Academy of Arts and Sciences 69, 189-264.

Tyzzer, E.E., Fabyan, M., 1920, Further studies on ‘‘Blackhead’’ in turkeys, with special reference to transmission by inoculation. Journal of Infectious Diseases 27, 207-239.

Van der Heijden, H.M.J.F., McDougald, L.R., Landman, W.J.M., 2005, High yield of parasites and prolonged *in vitro* culture of *Histomonas meleagridis*. Avian Pathology 34, 505-508.

Wernery, U., Kinne, J., 2002, Blackhead in Arabian red-legged partridge, stone curlew and spotted thick-knee (Dikkop). Falco 19, 19.

Wörheide, G., Nichols, S.A., Goldberg, J., 2004, Intragenomic variation of the rDNA internal transcribed spacers in sponges (Phylum *Porifera*): implications for phylogenetic studies. Molecular Phylogenetic Evolution 33, 816-830.

Zenner, L., Callait, M.P., Granier, C., Chauve, C., 2003, *In vitro* effect of essential oils from *Cinnamomum aromatocum*, *Citrus limon* and *Allium savitum* on two intestinal flagellates of poultry, *Tetratrichimonas gallinarum* and *Histomonas meleagridis*. Parasite 10, 153-157.

Chapter 5.2

Intragenomic variation in the internal transcribed spacer 1 region of *Dientamoeba fragilis* as a molecular epidemiological marker

A. Bart[1], H.M.J.F. van der Heijden[2], S. Greve[1], D. Speijer[3], W.J.M. Landman[2] and T. van Gool[1]

1. University of Amsterdam, Academic Medical Center, Department of Microbiology, Section Parasitology, Meibergdreef 9, 1105 AZ Amsterdam, the Netherlands
2. Animal Health Service Ltd, P.O. Box 9, 7400 AA Deventer, the Netherlands
3. University of Amsterdam, Academic Medical Center, Department of Medical Biochemistry, Amsterdam

Journal of Clinical Microbiology, 46 (2008) 3270-3275

Abstract

Dientamoeba fragilis is a parasite that has been recognized to be a causative agent of gastrointestinal symptoms. Because in most studies only some infected persons experience symptoms, it is possible that *D. fragilis* is a heterogeneous species with variants that display similar morphologies but different pathogenicities. The search for genetic variation in *D. fragilis* was based on the small-subunit rRNA gene, which was not found to be useful for molecular epidemiology. In this report, we describe the isolation and characterization of additional rRNA gene cluster sequences, the internal transcribed spacer 1 (ITS-1)–5.8S rRNA gene–ITS-2 region. For comparative purposes, we also isolated the ITS-1–5.8S rRNA gene–ITS-2 region of *Histomonas meleagridis*, a protozoan parasite of birds and a close relative of *D. fragilis*. This region was found to be highly variable, and 11 different alleles of the ITS-1 sequence could be identified. Variation in the ITS-1 region was found to be intragenomic, with up to four different alleles in a single isolate. So-called C profiles were produced from the ITS-1 repertoire of single isolates. Analysis of the C profiles of isolates from nonrelated patients identified several clearly distinguishable strains of *D. fragilis*. Within families, it was shown that members can be infected with the same or different strains of *D. fragilis*. In conclusion, the ITS-1 region can serve as a molecular epidemiological tool for the subtyping of *D. fragilis* directly from feces. This may serve as a means of studying the transmission, geographical distribution, and relationships between strains and the pathogenicity of this parasite.

Introduction

The protozoan *Dientamoeba fragilis* is found in the mucosal crypts of the large intestines of humans. For a long time, this parasite was ignored by most clinicians; but recently, evidence has been accumulating that *D. fragilis* is an important enteric pathogen associated with abdominal pain, bloating, diarrhoea, and irritable bowel syndrome (Johnson et al., 2004). Despite its global distribution and its apparent causative role in gastrointestinal complaints, very little is known about its pathogenicity, route of transmission, epidemiology, or genetics. This neglect of *D. fragilis* is partly due to the difficulties associated with the diagnosis of *D. fragilis* infection. The organism degenerates rapidly in passed stool and lacks a cyst stage, making microscopic detection possible only with freshly passed stool or by the use of fixatives and permanent stains. Recently, PCR-based methods that detect *D. fragilis* in a clinical sample without the need for prior culturing of the organism have been developed (Peek et al., 2004; Stark et al., 2005; Verweij et al., 2007). These methods make use of the multicopy, small-subunit (ssu) rRNA gene, the sequence of *D. fragilis* that has been the best studied to date. The ssu rRNA gene is useful for the sensitive and specific detection of *D. fragilis* by diagnostic PCR analysis, but due to a lack of variability in both symptomatic and asymptomatic individuals, this target is not suitable for epidemiological studies. Although heterogeneity in the *D. fragilis* ssu rRNA gene sequence, which results in the differentiation of genotypes 1 and 2, was reported (Peek et al., 2004), the latter genotype was found in only two cases and not in subsequent studies of larger groups of patients in the Netherlands (Peek et al., 2004), England (Windsor et al., 2006), and Australia (Stark et al., 2005b). This indicated that the ssu rRNA gene of *D. fragilis* displays insufficient variability to be used as a molecular epidemiological marker.

In an effort to detect genetic variations in *D. fragilis* that could be used as molecular epidemiological markers, we isolated and characterized part of the ribosomal gene cluster region containing internal transcribed spacer 1 (ITS-1), the 5.8S rRNA gene (5.8S gene), and ITS-2 of *D. fragilis* and the closely related avian parasite *Histomonas meleagridis*. The ITS-1–5.8S gene–ITS-2 region has been extensively used in phylogenetic studies and as a molecular epidemiological marker for other parasites. Concurrently, intragenomic variation in the ITS region of *D. fragilis* was observed by Windsor et al. (2006), who dismissed this target as a marker for strain subtyping, as sequence traces derived from direct amplification would be too complex. However, in the closely related avian parasite *Histomonas meleagridis*, the complexity of ITS sequencing electropherograms was successfully reduced by a method called “C profiling” (Van der Heijden et al., 2006).

The present paper describes the identification of 11 different variants of the ITS-1 region of *D. fragilis*, confirming and extending the results obtained by Windsor et al. (2006). As observed with *Histomonas*, analysis of the C nucleotide residues in these complex chromatograms produced chromatograms that were

reproducible and easy to interpret. This method could be applied to generate a molecular epidemiological marker of *D. fragilis* directly from stool specimens that can be used to study the transmission and geographical distribution of strains, the relationships between strains, and the pathogenicity of this parasite.

Materials and Methods

Stool specimens and DNA extraction. Intestinal parasites, including *D. fragilis*, were detected by the triple feces test (Van Gool et al., 2003). Stool specimens that were *D. fragilis* positive by microscopy were collected from patients presenting with gastrointestinal symptoms in routine clinical practice of the Academic Medical Center of Amsterdam. In addition, stool specimens microscopically positive for *D. fragilis* from asymptomatic carriers with no gastrointestinal complaints during the previous 2 months were included. Details of the processing of the fecal samples and subsequent DNA isolation were described earlier (Peek et al., 2004). A limiting dilution of *D. fragilis* trophozoites was obtained by 10-fold serial dilution of freshly passed stool samples from two patients in phosphate-buffered saline, followed by DNA extraction. *D. fragilis* genotype 2 DNA was isolated from strain Bi/pa (ATCC 30948). *Trichomonas vaginalis* was obtained from a vaginal swab specimen, followed by culturing in Trypticase-yeast extract-maltose. *Histomonas meleagridis* was cultured from the cecum of an infected turkey (Van der Heijden et al., 2006). DNA was isolated from the *T. vaginalis* and *H. meleagridis* cultures as described above for the *D. fragilis* samples.

PCR method. The design of forward primer ssu2 (5'-GGAATCCCTTGTAA ATGCGT-3') was based on the sequences of the ssu rRNA genes of *D. fragilis* (GenBank accession number U37461), *H. meleagridis* (GenBank accession number AF293056), and *T. vaginalis* (GenBank accession number AY677680). Reverse primer lsu1 (5'-AGTTCAGCGGGTCTTCCTG-3') was complementary to a conserved region in the 5' end of the large-subunit (lsu) rRNA genes of *Trichomonad* species. The ssu2 and lsu1 primer combination was used for PCR amplification of the ITS-1–5.8S gene–ITS-2 region. Reverse primer 5.8s1 (5'-TGTGAGGAGCCAAGACATCC-3') was complementary to the 5' end of the 5.8S rRNA gene of *D. fragilis*. The ssu2 and 5.8s1 primer combination was used for PCR amplification of the ITS-1 region. For each series of samples, a negative control containing no input DNA was added. The PCR mixture (50 µl) contained 20 µl of DNA solution, 100 ng of each primer, 0.5 mM of a deoxynucleoside triphosphate mixture, 5 µl of 10µl PCR buffer (Promega), 6 µl of 25 mM $MgCl_2$, 5 µl of bovine serum albumin (5 mg/ml), 5 µl of α-casein (20 mg/ml) to relieve PCR inhibition by fecal substances, and 0.2 U *Taq* polymerase (Promega). After 3 min of initial denaturation at 94 °C, 40 cycles of denaturation at 94 °C for 1 min, annealing

at 52 °C for 1 min, and extension at 72 °C for 1 min were performed, followed by a final extension at 72 °C for 5 min. The amplification products were size fractionated on 1% agarose gels and visualized by ethidium bromide staining.

The sensitivity of the assay was assessed in several PCR experiments with a range of dilutions (in Tris-EDTA buffer with bacteriophage lambda DNA) of a control pGEMT vector containing a single copy of ITS-1. It could be established how many copies were minimally needed under our assay conditions to still obtain a PCR signal. A minimum of 10 copies (representing one individual parasite containing 11 copies) was needed.

Cloning and sequencing. One microliter of the PCR product was ligated in the PCR cloning vector pGEMTeasy, as described by the manufacturer (Promega). After transformation into *Escherichia coli* DH5α, individual clones were tested for the presence of an insert by a colony PCR for 25 cycles with either the primer combination ssu2 and lsu1 (specific for the ITS-1-5.8S gene-ITS-2 region) or the primer combination ssu2 and 5.8s1 (specific for the ITS-1 region). Individual clones with an insert were subjected to sequencing with primer ssu2. For direct sequencing of the ITS-1 PCR products, an Applied Biosystems Prism 310 dye terminator fluorescence-based genetic analyzer was used. Homology searches were done by using the BLAST program with the default settings (available at http://www.ncbi.nlm.nih.gov/blast/). Multiple-sequence alignments, C profiles, and insertion/deletion (indel) analysis were performed by using CodonCode software (CodonCode Corporation, Dedham, MA).

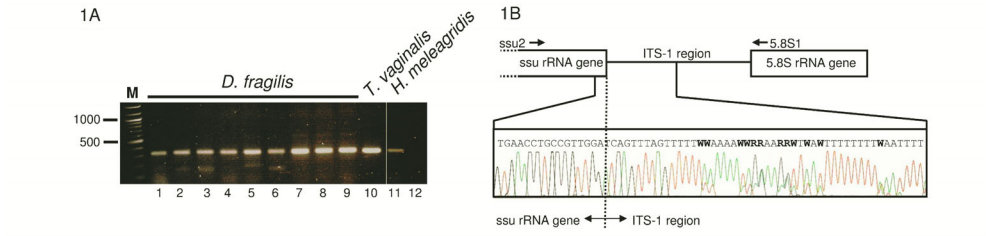
Nucleotide sequence accession number. The nucleotide sequence data reported in this study for the *D. fragilis* ITS-1-5.8S gene-ITS-2 region appear in the European Molecular Biology Laboratory (EMBL) GenBank database under accession number DQ167586.

Results

Isolation of ITS-1-5.8S gene-ITS-2 region of *D. fragilis* and related species. To obtain sequence information on the ITS-1-5.8S gene-ITS-2 ribosomal gene region of *D. fragilis*, several PCR primer pairs were designed to amplify the sequences between the 3' end of the ssu rRNA gene and the 5' end of the lsu rRNA gene. The forward primers were complementary to the published ssu rRNA sequences of *D. fragilis*, *H. meleagridis*, and *T. vaginalis* (GenBank accession numbers U37461, AF293056, and AY677680 respectively) and contained several nucleotides at their 3' ends that did not match the ssu rRNA gene sequences of other parasites prevalent in the human gut (data not shown). We therefore designed reverse primers in a conserved region at the utmost 5' end of the lsu rRNA gene, based on

published lsu rRNA gene sequences of trichomonads (data not shown). Only the primer combination ssu2 and lsu1 amplified a product of approximately 500 bp from DNA extracted from human feces containing *D. fragilis*, cultured *T. vaginalis* parasites, and cultured *H. meleagridis* parasites. These PCR products were excised from the gels, cloned, and sequenced. Sequence analysis showed that we indeed successfully cloned the ITS-1-5.8S gene-ITS-2 region of all three species. Both the ITS-1 region and the ITS-2 region of *D. fragilis* and *H. meleagridis* were found to be extremely AT rich (~94%), while the 5.8S rRNA genes had an AT content of approximately 60%. The putative 5' and 3' ends of the ITS-1, 5.8S rRNA gene, and ITS-2 were based on homology with 5.8S rRNA genes of other trichomonads (data not shown) and abrupt changes in the AT content of the sequence. The 5.8S rRNA genes of *D. fragilis* and *H. meleagridis* appeared to be truncated at the 3' end and were approximately 45 nucleotides shorter than those of the other trichomonads (data not shown).

The ITS-1 sequence of *D. fragilis* is variable. The PCR for amplification of the complete ITS-1-5.8S gene-ITS-2 region appeared to be too inefficient for the study of the genetic variability of *D. fragilis* parasites directly from human feces. We therefore focused on the ITS-1 region and developed a PCR using ssu rRNA-specific primer ssu2 and 5.8S rRNA gene specific primer 5.8s1. This PCR was able to efficiently amplify the 3' end of the ssu rRNA and the complete ITS-1 region of *D. fragilis* directly from fecal DNA and from cultured *T. vaginalis* and *H. meleagridis* parasites (Figure 1A). Analysis of the sequences of the PCR products from *D. fragilis* showed a full match with the published ssu rRNA gene sequence and an aberrant ITS-1 sequence, with many double base calls detected in the sequencing chromatograms (Figure 1B). This suggested that the ITS-1 sequence of *D. fragilis* is heterogeneous and that several ITS-1 variants were present in a single fecal specimen. An aberrant ITS-1 sequence with many double base calls was also observed in *H. meleagridis* but not in two clinical isolates of *T. vaginalis* (data not shown). The PCR products of *D. fragilis* from fecal samples from eight nonrelated individuals were cloned to further delineate the sequence heterogeneity within the ITS-1 region. Full-length sequencing of individual clones showed that among these eight fecal samples, six samples each contained three ITS-1 alleles, while two and four alleles were detected in one fecal sample each. A total of 11 different ITS-1 alleles could be identified. On the basis of the divergence in the 3' part of the sequence, it was possible to separate these 11 ITS-1 alleles into two groups: alleles 1 to 5 and alleles 6 to 11 (Figure 1c). Within these two groups of ITS-1 alleles, sequence variation was limited to single A/T indels. In addition to the sequences of *D. fragilis* genotype 1, we also analyzed the ITS-1 region of *D. fragilis* genotype 2 using the same ssu2 and 5.8s1 primer combination. Similar to genotype 1, the ITS-1 region of genotype 2 displayed sequence ambiguity, indicating heterogeneous ITS-1 sequences.



1C

```
type 1          -GTT-TAGTT TTTTT---AA AAATACAAAC ATTAATTTTT TTT--TAAAT TTTAAAAAT- TATACA---- ---AAATTTT T---TAAAAA AAA-TTTAAA TTTTTAGA-
type 2          -GTT-TAGTT TTTA----AA AAATACAAAC ATTAATTTTT TTT--TAAAT TTTAAAAAT- TATACA---- ---AAATTTT T---TAAAAA AAA-TTTAAA TTTTTAGA-
type 4          -GTT-TAGTT TTTTT---AA AAATACAAAC ATTAATTTTT TTT--TAAAT TTTAAAAAT- TATACA---- ---AAATTTT T---TAAAAA AAAATTTAAA TTTTTAGA-
type 5          -GTT-TAGTT TTTT----AA AAATACAAAC ATTAATTTTT TTT--TAAAT TTTAAAAAT- TATACA---- ---AAATTTT T---TAAAAA AAA-TTTAAA TTTTTAGA-
type 6          -GTT-TAGTT TTTTA----A AAATACAAAC ATTAATTTTT TT---AAAAT TTTAAAAACA TATACATAAA ATTAAATTTT T---TTTTAA AAA------- ATTTTAGA-
type 7          -GTT-TAGTT TTTAA----A AAATACAAAC ATTAATTTTT TT---AAAAT TTTAAAAACA TATACATAAA ATTAAATTTT T---TTTAAA AAA------- ATTTTAGA-
type 8          -GTT-TAGTT TTTAA----A AAATACAAAC ATTAATTTTT TTT--AAAAT TTTAAAAACA TATACATAAA ATTAAATTTT T---TTTAAA AAA------- ATTTTAGA-
type 9          -GTT-TAGTT TTTTA----A AAATACAAAC ATTAATTTTT T----AAAAT TTTAAAAACA TATACATAAA ATTAAATTTT T---TTTTAA AAA------- ATTTTAGA-
type 10         -GTT-TAGTT TTTTA----A AAATACAAAC ATTAATTTTT TTT--AAAAT TTTAAAAACA TATACATAAA ATTAAATTTT T---TTTAAA AAA------- ATTTTAGA-
type 11         -GTT-TAGTT TTTAA----A AAATACAAAC ATTAATTTTT TTT--TAAAT TTTAAAAACA TATACATAAA ATTAAATTTT T---TTTAAA AAA------- ATTTTAGA-
genotype 2      -GTT-TAGTT TTTTTT--TA AAATACAAAC ATTAATTTTT TG---ATATT TTTTAAAATT TATACATAAA AAAGAATTTT T---TTTAAA ---------- --CTTAGA-
H. meleagridis --TT-TAGTT TTTTATTTAA AAAAAAAAAC AATAATTTTT TTTATAAAAT TTAAACAATT TATACAAAAT TCAAAATCAA TAAATTTCAA AAA------- ---TTAAA-
T. vaginalis    AGTTCTAGTT TT-------- AATAACTAAC ACCAACTTCT TTTATTAAAC ---AAAAACC AATACAAAAT TAAAAACTAA C----TTCAT CAA------- -----AAAC
```

Figure 1. (A) PCR amplification of the ITS-1 region of *D. fragilis* directly from human feces (lanes 1 to 9) and from cultured *T. vaginalis* (lane 10) and *H. meleagridis* (lane 11) parasites. Lane 12, results of PCR without template. The size marker (lane M) is a 100-bp ladder. (B) Schematic representation of the region amplified by primers ssu2 and 5.8s1. The partial sequencing chromatogram of the 3' end of the ssu RNA gene and the 5' end of the ITS-1 region of *D. fragilis* illustrates the ambiguities and double base calls in the ITS-1 sequence. (C) Sequence alignment of the 11 different cloned ITS-1 regions of *D. fragilis* from fecal samples from eight patients and cloned ITS-1 regions of *D. fragilis* genotype 2, *T. vaginalis*, and *H. meleagridis*. The *D. fragilis* ITS-1 regions can be divided into two groups (types 1 to 5 and types 6 to 11, respectively) on the basis of the presence of the C residue at position 52 and sequence divergence in the 3' part of the ITS-1 region.

The cloned ITS-1 region of *D. fragilis* genotype 2 also showed a high AT content, but its sequence was different from the ITS-1 sequences of *D. fragilis* genotype 1 isolates (Figure 1C, genotype 2).

The variability of the ITS-1 sequence can be used as a molecular epidemiological marker for *D. fragilis*. The presence of multiple ITS-1 alleles of *D. fragilis* in a single human fecal sample resulted in a complex and aberrant chromatogram during sequence analysis (Figure 1B). A reduction of this complex chromatogram by deleting the peaks for the A, T, and G nucleotides gave a pattern

of C nucleotides only that was relatively easy to interpret (Figure 2). In this so-called C profile, each C residue is represented by multiple adjoining peaks. The multiple peaks for each C residue are caused by A/T nucleotide indels between the individual ITS-1 alleles. The relative heights of the individual peaks of the C profile depend on the relative abundance of the different ITS-1 alleles within a fecal sample. For example, the first two adjacent peaks in the C profiles shown in Figure 2A correspond to the C residue at position 52 of ITS-1 types 6 to 11 (Figure 1C). In the C profile of patient samples 1 and 2, these first two peaks were missing. This indicates that in these samples, only ITS-1 sequences missing the C residue at position 52 are present (Figure 1C, alleles [types] 1 to 5). The reproducibility of the C profile was evaluated by repeating the DNA extraction and sequence analysis of three fecal samples containing *D. fragilis*. This demonstrated that the C profile was indeed reproducible for a particular sample (data not shown).

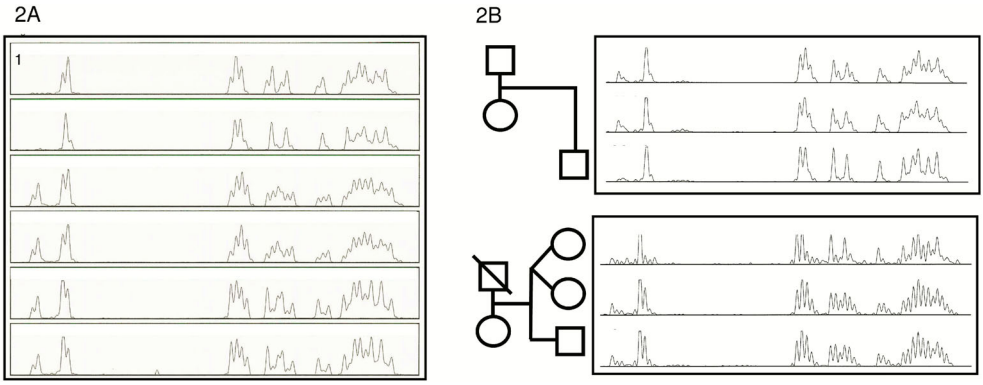


Figure 2. (A) C profiling of the *D. fragilis* ITS-1 regions directly from fecal specimens of nonrelated patients. Rows 1, 2, 3, and 5, the four different C profiles identified; row 4, C profile from a patient similar to the C profile in row 3; row 6, C profile from a patient similar to the C profile in row 5. (B) C profiling of the *D. fragilis* ITS-1 region in families. C profiles from parents asymptomatically infected with *D. fragilis* and their symptomatically infected son are shown in the top panel. The C profiles of twin sisters and their brother are shown in the bottom panel. All three children were asymptomatically infected with *D. fragilis*.

Analysis of fecal samples containing *D. fragilis* from samples from 10 nonrelated patients showed four different C profiles, which were present in one, two, two, and five samples, respectively (Figure 2A). In addition, we tested fecal samples containing *D. fragilis* from the members of two families. For the family whose results are shown in the top panel of Figure 2B, both the father and the mother were infected with *D. fragilis* but showed no clinical signs. In contrast, their infected son (age, 6 years) did have gastrointestinal complaints. The samples from both parents displayed very similar C profiles, while the sample from their son had

a different C profile. Interestingly, the C profile of the sample from the son (C profile 9) was very similar to the C profile of the sample from another patient (Figure 2A, C profile 2). In the family whose results are shown in the bottom panel of Figure 2B, the twin sisters (ages, 4 years) and their brother (age, 7 years) were asymptomatically infected with *D. fragilis*. The twin sisters were infected with different *D. fragilis* variants, while their brother was infected with the same variant as one of his sisters.

The ITS-1 variation in *D. fragilis* is intragenomic. The results presented above show that a fecal sample from an infected individual contains different *D. fragilis* ITS-1 alleles. This can be explained either by the presence of multiple *D. fragilis* strains within a single fecal sample or by intragenomic variation of the ITS-1 alleles within a single *D. fragilis* trophozoite, or by a combination of both possibilities. To discriminate between these possibilities, a limiting dilution of freshly passed stool samples from two patients containing *D. fragilis* trophozoites was made. If different strains with different genotypes were present with different relative abundances, then the profile would change as the parasite populations were diluted out. DNA was extracted from each dilution step, and a C profile was produced from the nondiluted sample and from the 10^1-, 10^5-, and 10^6-fold dilutions (Figure 3).

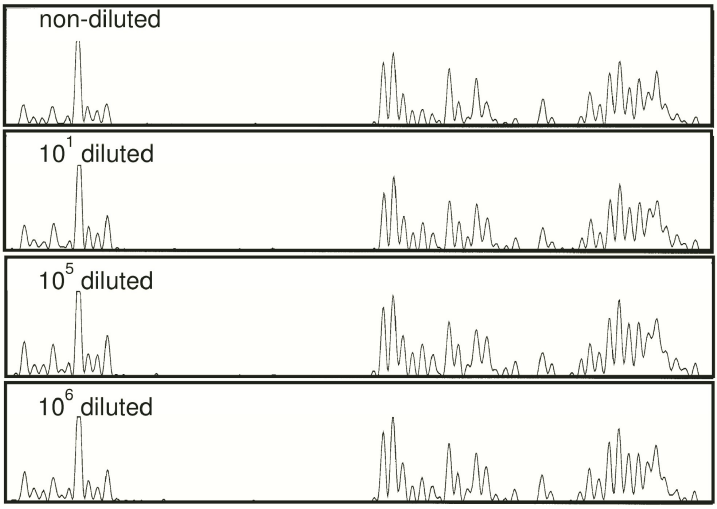


Figure 3. Limiting dilution of a fecal sample containing *D. fragilis* trophozoites. The C profiles for a nondiluted sample and samples diluted 10^1, 10^5, and 10^6 times are shown. No significant differences between nondiluted and diluted samples were observed.

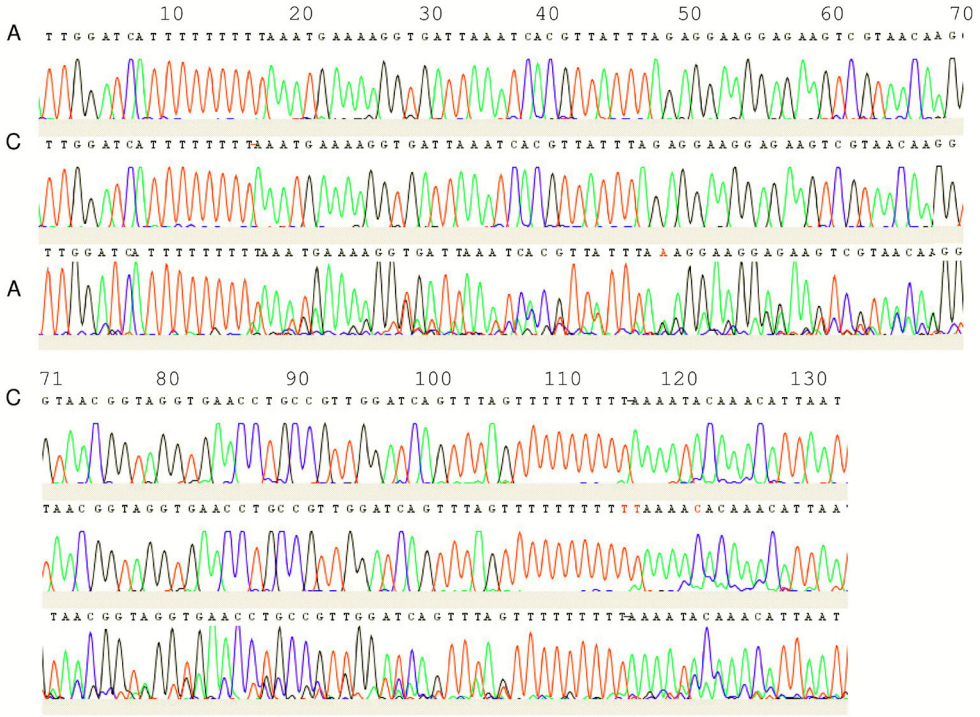


Figure 4. Sequence chromatograms of two individual clones (clones a and b; top two rows) derived from the PCR product of strain Bi/pa (genotype 2) and the PCR product itself (c; bottom row). CodonCode predicts an indel at position 17, which corresponds to a difference in the stretch of T residues at about position 10 between the two clones (clone a, eight T residues; clone b, nine T residues). From this point onward, each peak is preceded by a smaller identical peak. Variation in the stretch of T residues at about position 110 (clone a, 9 T residues; clone b, 11 T residues) and the T-C transition at position 120 result in even more complexity after position 110.

In the 10^6-fold dilution, which corresponded to the detection limit, no significant change in the C profile compared to that for the nondiluted sample could be observed. Similar results were obtained with the sample from the second patient (data not shown). It is highly unlikely that more than one subtype of *D. fragilis* was present in both stool samples with equal abundances and that at the 10^6-fold dilution the complete array of ITS-1 types would still be present. The weight of the evidence therefore indicates that ITS-1 sequence variation is of intragenomic origin only.

Similarly, variation in the ITS of strain Bi/pa (genotype 2) was observed. Since this culturable strain represents a clonal population, variation in this isolate must be intragenomic. Direct sequencing of a PCR product obtained from this strain yielded

a complex chromatogram (Figure 4), for which the CodonCode program predicted heterozygous indels. The sequencing of individual clones derived from the PCR product itself confirmed that the observed chromatogram was the result of sequence heterogeneity, including indel events in mononucleotide stretches in the sequence (Figure 4).

Discussion

The search for genetic variation in *D. fragilis* that can be used for molecular epidemiology has been the topic of several recent papers by research groups from the United Kingdom, The Netherlands, and Australia (Johnson and Clark, 2000; Peek et al., 2004; Stark et al., 2005a, 2005b; Windsor et al., 2004). These studies focused on the detection of sequence heterogeneity in the ssu rRNA gene of *D. fragilis*, which, to date, is the only sequence information available for this organism. Surprisingly, all but 1 of the 154 *D. fragilis* ssu rRNA sequences obtained from clinical samples showed no heterogeneity, and all sequences were assigned to genotype 1. The genotype 2 ssu rRNA gene was found in only one clinical sample, and its sequence was identical to that of *D. fragilis* strain Bi/pa (ATCC 30948). From these observations, it was concluded that *D. fragilis* appears to be a clonal species and that the genotype 1/2 variation could not be used for epidemiological studies. In the current study we isolated the ITS-1-5.8S gene-ITS-2 region of *D. fragilis* and examined it for genetic variation. For reasons of comparison. the ITS-1-5.8S gene-ITS-2 region of the closely related bird parasite *H. meleagridis* was also isolated. The ITS regions of the ribosomal gene cluster have no function in the mature ribosome and are thus likely to sustain much more sequence variation than coding regions. ITS regions are among the markers that are the most frequently used to study intra- and interspecific evolution in all kinds of organisms, including human parasites (Chilton and Gasser, 1999; Kumar and Suhkla, 2005; Olivier et al., 2001). Analysis of the ITS-1-5.8S gene-ITS-2 region of *D. fragilis* showed that the 5.8S rRNA genes of *D. fragilis* and *H. meleagridis* are surprisingly short (111 bp) compared to the lengths of the 5.8S rRNA genes of other trichomonads (~150 bp). The high degree of homology between the 5.8S rRNA genes of *D. fragilis* and *H. meleagridis* further substantiates the observation that these organisms are closely related (Johnson et al, 2000; Silberman et al., 1996). The ITS-1 and ITS-2 sequences were extremely AT rich and showed extensive variation in *D. fragilis*, both within and between clinical isolates. Cloning and sequencing of individual ITS-1 regions from *D. fragilis* revealed 11 different ITS-1 alleles which could be separated into two different groups, confirming and extending the observations of Windsor et al. (2006).

Up to four different *D. fragilis* ITS-1 sequences could be observed in a single fecal specimen. Although this could indicate polyparasitism, in which the host is

infected with multiple parasite variants (Olivier et al., 2001), limiting-dilution experiments with intact trophozoites demonstrated that the variation in the ITS-1 region is intragenomic. *H. meleagridis* also shows this intragenomic heterogeneity of the ITS-1 region (Van der Heijden et al., 2006), while the two clinical isolates of *T. vaginalis* were devoid of ITS-1 sequence variation. The intragenomic heterogeneity of the ITS regions has already been observed in many different organisms. The extent varies greatly between taxa and is thought to arise by mechanisms such as slipped-strand mispairing events (Wörheide et al., 2004).

With the C-profiling technique, we found four different types of *D. fragilis* parasites among 10 randomly chosen, nonrelated patients. This suggests that the variability that exists in *D. fragilis* is sufficient for the use of this simple technique for the generation of a molecular epidemiological marker. This was substantiated by the analysis of two families infected with *D. fragilis*. In the first family, both parents were asymptomatically infected with the same type, while their 6-year-old son had gastrointestinal complaints and was infected with a different, possibly more virulent strain also detected in another symptomatic subject. Twin sisters of the second family were infected with different types, even though these girls spent most of their time together and visited the same day care center. Interestingly, their brother was infected with the same type as one of his sisters. Clearly, more data from large groups of asymptomatic individuals and patients with different symptomatologies are needed to establish a possible link between infection with a particular *D. fragilis* type and the clinical outcome of the infection. Moreover, more types may exist in different geographical locations.

The specificity of the ITS-1 PCR was not investigated in detail in this paper. However, the 204 bp of the ssu rRNA gene sequence that is part of the ~300-bp ITS-1 PCR amplicon was always found to be identical to the sequence of the 3' end of the *D. fragilis* genotype 1 ssu rRNA. DNA was not amplified from other (gut) protozoa, including *Entamoeba dispar*, *Entamoeba coli*, *Entamoeba hartmanni*, *Entamoeba histolytica*, *Blastocystis hominis*, *Saccharomyces cerevisiae*, and *Encephalitozoon hellem*, or from smear-negative controls (data not shown).

Our study clearly shows that the intragenomic variation of the ITS-1 region of *D. fragilis* can be used as a molecular epidemiological marker. Variation in this region could be directly analyzed from human feces by PCR amplification and sequencing without the need for prior culturing of the parasite. Techniques such as restriction fragment polymorphism analysis and single-strand conformation polymorphism analysis have also been successfully used for the detection of ITS sequence variations in human parasites (Chalmers et al., 2005; Cupolillo et al., 2003; Kumar and Shukla, 2005; Nejsum et al., 2005; Schönian et al., 2001). However, due to the extreme AT content and intragenomic variation of the *D. fragilis* ITS sequences, these techniques did not give satisfying results in our hands (data not shown). By analyzing only the peaks for C nucleotides from a sequencing chromatogram of the ITS-1 region of *D. fragilis* amplified directly from

human feces, a profile that is relatively easy to interpret is obtained. Most research laboratories now have access to fluorescencebased sequence analyzers and should be able to create such C profiles. The ITS-1 C profile can be considered a fingerprint of *D. fragilis* which is found to be highly reproducible. This technique can now be used to discriminate between persistent infection and reinfection after drug treatment and for molecular epidemiological studies that are aimed at investigating the geographical distribution of *D. fragilis* strains, their transmission, and, most importantly, the possible correlation between various parasite strains and different degrees of virulence.

Acknowledgements

We thank Ron Peek for initiating this study and Youssef Hsaini for expert technical assistance.

References

Chalmers, R.M., Ferguson, C., Cacciò, S., Gasser, R.B., Abs-El Osta, Y.G., Heijen, L., Xiao, L., Elwin, K., Hadfield, S., Sinclair, M., Stevens, M., 2005, Direct comparison of selected methods for genetic categorisation of *Cryptosporidium parvum* and *Cryptosporidium hominis* species. International Journal of Parasitology 35, 397-410.

Chilton, N.B., Gasser, R.B., 1999, Sequence differences in the internal transcribed spacers of DNA among four species of hookworm (Ancylostomatoidea: Ancylostoma). International Journal of Parasitology 29, 1971-1977.

Cupolillo, E., Brahim, L.R., Toaldo, C.B., De Oliveira-Neto, M.P., De Brito, M.E.F., Falqueto, F., De Farias Naiff, M., Grimaldi, G., 2003, Genetic polymorphism and molecular epidemiology of *Leishmania* (*Viannia*) *braziliensis* from different hosts and geographic areas in Brazil. Journal of Clinical Microbiology 41, 3126-3132.

Johnson, E.H., Windsor, J.J., Clark, C.G., 2004, Emerging from obscurity: biological, clinical, and diagnostic aspects of *Dientamoeba fragilis*. Clinical Microbiology Reviews 17, 553–570.

Johnson, J.A., Clark, C.G., 2000, Cryptic genetic diversity in *Dientamoeba fragilis*. Journal of Clinical Microbiology 38, 4653-4654.

Kumar, M., Shukla, P.K., 2005, Use of PCR targeting of internal transcribed spacer regions and single-stranded conformation polymorphism analysis of sequence variation in different regions of rRNA genes in fungi for rapid diagnosis of mycotic keratitis. Journal of Clinical Microbiology 43, 662-668.

Nejsum, P., Parker, E.D., Frydenberg, J., Roepstorff, A., Boes, J., Haque, R., Astrup, I., Prag, J., Skov Sørensen, U.B., 2005, Ascariasis is a zoonosis in Denmark. Journal of Clinical Microbiology 43, 1142-1148.

Olivier, C., Van de Pas, S., Lepp, P.W., Yoder, K., Relman, D.A., 2001, Sequence variability in the first internal transcribed spacer region within and among Cyclospora species is consistent with polyparasitism. International Journal of Parasitology 31, 1475-1487.

Peek, R., Reedeker, F.R., Van Gool, T., 2004, Direct amplification and genotyping of *Dientamoeba fragilis* from human stool specimens. Journal of Clinical Microbiology 42, 631-635.

Schönian, G., Schnur, L., El Fari, M., Oskam, L., Kolesnikov, A.A., Sokolowska-Köhler, W., Presber, W., 2001, Genetic heterogeneity in the species Leishmania tropica revealed by different PCR-based methods. Transactions of the Royal Society of Tropical Medicine and Hygiene 95, 217-224.

Silberman, J.D., Clark, C.G., Sogin, M.L., 1996, *Dientamoeba fragilis* shares a recent common evolutionary history with the trichomonads. Molecular and Biochemical Parasitology 76, 311-314.

Stark, D., Beebe, N., Marriott, D., Ellis, J., Harkness, J., 2005a, Detection of *Dientamoeba fragilis* in fresh stool specimens using PCR. International Journal of Parasitology 35, 57-62.

Stark, D., Beebe, N., Marriott, D., Ellis, J., Harkness, J., 2005b, Prospective study of the prevalence, genotyping, and clinical relevance of *Dientamoeba fragilis* infections in an Australian population. Journal of Clinical Microbiology 43, 2718-2723.

Van der Heijden, H.M.J.F., Landman, W.J.M., Greve, S., Peek, R., 2006, Genotyping of *Histomonas meleagridis* isolates based on internal transcribed spacer-1 sequences. Avian Pathology 35, 330-334.

Van Gool, T., Weijts, R., Lommerse, E., Mank, T.G., 2003, Triple faeces test: an effective tool for detection of intestinal parasites in routine clinical practice. European Journal of Clinical Microbioly and Infectious Diseases 22, 284-290.

Verweij, J.J., Mulder, B., Poell, B., Van Middelkoop, D., Brienen, E.A.T., Van Lieshout, L., 2007, Real-time PCR for the detection of *Dientamoeba fragilis* in fecal samples. Molecular and Cellular Probes 21, 400-404.

Windsor, J.J., Clark, C.G., MacFarlane, L., 2004. Molecular typing of *Dientamoeba fragilis*. British Journal of Biomedical Science. 61, 153-155.

Windsor, J.J., MacFarlane, L., Clark, C.G., 2006, Internal transcribed spacer dimorphism and diversity in *Dientamoeba fragilis*. Journal of Eukaryotic Microbiology 53, 188-192.

Wörheide, G., Nichols, S.A., Goldberg, J., 2004, Intragenomic variation of the rDNA internal transcribed spacers in sponges (phylum Porifera): implications for phylogenetic studies. Molecular Phylogenetics and Evolution 33, 816-830.

Chapter 5.3

Molecular identification and phylogenetic relationships of Trichomonad isolates of galliform birds inferred from nuclear small subunit rRNA gene sequences

C. Mantini[1], J. Dalia-Cornette[1], S. Noda[2], H.M.J.F. van der Heijden[3], E. Dei-Cas[4], W.J.M. Landman[3], M. Ohkuma[2] and E. Viscogliosi[1]

1. Université Lille, Institut Pasteur de Lille, Inserm, U457, 1 rue du Professeur Calmette, BP 245, 59019 Lille cedex, France
2. Institute of Physical and Chemical Research RIKEN, Ecomolecular Biorecycling Science Research Team, Wako, Saitama 351-0198, Japan
3. Animal Health Service Ltd, P.O. Box 9, 7400 AA Deventer, the Netherlands
4. Université Lille, Insitut Pasteur de Lille, Laboratoire d'Ecologie du Parasitisme (EA 3609), 1 rue du Professeur Calmette, BP 245, 59019 Lille cedex, France

Submitted to Journal of Eukaryotic Microbiology

Abstract

Histomonas meleagridis is the aetiological agent of histomonosis, also called blackhead disease. Recently, genotyping based on polymerase chain reaction and sequencing of Internal Transcribed Spacer-1 sequences was applied to various isolates originating from fowl. Three genotypes were described: types I and II isolates were associated with clinical disease and probably derived from *H. meleagridis* whereas type III isolates were non-pathogenic and likely corresponded to *Parahistomonas wenrichi* according to its peculiarities described in previous studies. However, this latter species has never been characterized at the molecular level and its phylogenetic relationships with other parabasalids remained hypothetical. To confirm the identification of these isolates, nuclear small subunit rRNA gene sequences were obtained by polymerase chain reaction from representatives of types I, II, and III. These new sequences were analyzed in a broad phylogeny including 64 other parabasalid sequences. From our phylogenetic trees, we confirm that types I and II isolates are closely related, if not identical, to *H. meleagridis*. Type III isolates described as representatives of *P. wenrichi* share a common ancestor with *H. meleagridis* and both species cluster together with high support. This grouping is in agreement with morphological similarity observed between these two taxa and suggests that speciation leading to these two species inhabiting the same hosts and ecological niche, occurs recently in birds. This speciation was likely followed by loss of pathogenicity in *P. wenrichi*. Moreover, both taxa group with *Dientamoeba fragilis* and *Tritrichomonas foetus*, the latter of which exhibits a complex cytoskeleton. This clustering reinforces the hypothesis that *Histomonas*, *Dientamoeba*, and *Parahistomonas* which exhibit more simple ultrastructure, have secondarily lost some of their cytoskeletal structures in the course of evolution.

Introduction

Parabasalia (Adl et al., 2005) or commonly parabasalids, describe a large and diverse monophyletic group of anaerobic flagellated protists, whose common features include one or more parabasal apparatus consisting of a parabasal body (Golgi complex) and a parabasal filament, the presence of hydrogenosomes (modified mitochondria), and a special type of closed mitosis called cryptopleuromitosis (Brugerolle, 1976; Brugerolle and Lee, 2001; Honigberg and Brugerolle, 1990). Phylogenetic relationships among the parabasalids were first proposed on the basis of morphological differences mostly linked to the structure and development of the cytoskeleton (Brugerolle, 1976; Honigberg, 1963). More recently, nuclear small subunit (SSU) rRNA gene sequences comparison was extensively used in parabasalid reconstructions (for recent references see Carpenter and Keeling, 2007; Dufernez et al., 2007; Gerbod et al., 2002; Hampl et al., 2004, 2006, 2007; Keeling, 2002; Noël et al., 2007; Ohkuma et al., 2005). The SSU rRNA analyses were globally incongruent with the morphology-based classification of this group (Brugerolle, 1976; Honigberg, 1963) and many authors pointed out the need to revise parabasalid systematics. Adl et al. (2005) were the last to propose a new classification of parabasalids based on both morphological and molecular data and divided the group into four orders: Trichomonadida, Cristamonadida, Spirotrichonymphida, and Trichonymphida. However, the classification of these authors is still incongruent with recent large-scale parabasalid phylogenies in several respects such as the polyphyly / paraphyly of the order Trichomonadida (Dufernez et al., 2007; Hampl et al., 2006, 2007; Noël et al., 2007).

The order Trichomonadida (Adl et al. 2005) includes most of the taxa belonging to the Monocercomonadidae family. As proposed in the morphology-based classification (Brugerolle, 1976; Honigberg, 1963), this family is composed of the simplest parabasalids in term of structure and was thought to represent the ancestral morphology of the parabasalids. However, as stated in several phylogenetic studies (Edgcomb, 1998; Gerbod et al., 2001, 2002; Hampl et al., 2004, 2006, 2007; Keeling, 2002; Noël et al., 2007; Ohkuma et al., 2005), the Monocercomonadidae form a polyphyletic group and none of the Monocercomonadidae lineage is located at the root of the parabasalid tree. These data implied that the relative morphological simplicity of the Monocercomonadidae must have arisen through the secondary reduction or loss of some cytoskeletal structures. Such reduction of the cytoskeleton in the course of evolution was for instance obvious for the Monocercomonadidae *Histomonas meleagridis* (Gerbod et al., 2001). This species which has four basal bodies, three of them being barren, and a reduced microtubular cytoskeleton called axostyle-pelta complex (Rybicka et al., 1972) was shown to be derived from a more complex form, the Trichomonadidae *Tritrichomonas* as revealed by the possession of four flagella, a well-developed

axostyle-pelta complex, a large striated root, the costa, and a complex locomotory organelle, the undulating membrane (Honigberg et al., 1971).

Histomonas meleagridis which is phylogenetically related to *Dientamoeba fragilis* (Gerbod et al., 2001), a non-flagellated Monocercomonadidae considered pathogenic to humans, parasitizes many galliform and some anseriform birds, and is the aetiological agent of a type of enterohepatitis termed histomonosis or "blackhead" disease (Lund, 1969; McDougald, 1997; Reid, 1967; Smith, 1895; Tyzzer, 1920). Two forms of *H. meleagridis* have been described: an aflagellated "tissue form" and a flagellated "lumen-dwelling form" possessing one anterior flagellum which is lost during the invasion of the host's tissues (Lund et al., 1967). However, the identification of this parasite in the caecal lumen of chickens, turkeys, and pheasants was confused by findings of a larger cell type equipped with 4 flagella, in addition to the predominant smaller uniflagellate forms corresponding to *H. meleagridis* (Tyzzer, 1934; Wenrich, 1943). Wenrich (1943) considered the larger 4-flagellate forms as belonging to *H. meleagridis* and was inclined to view them as representing "adult" stages of the parasite. Subsequently, Lund (1963) created a new species *wenrichi* in the genus *Histomonas* for these larger 4-flagellate forms which were considered as non-pathogenic for fowl. Ultimately, Honigberg and Kuldova (1969) supported the taxonomic considerations by Lund (1963) while suggesting the creation of a new genus, *Parahistomonas*, for these larger organisms on the basis of the number of flagella.

As stated above, *Parahistomonas wenrichi* was placed in a new genus mainly on the base of a restricted number of morphological characters (size and number of flagella). Thus, the validity and classification of this taxon has to be confirmed by molecular data. Recently, a novel genotyping method based on polymerase chain reaction and sequencing of Internal Transcribed Spacer-1 (ITS-1) domains was applied to various isolates potentially identified as *H. meleagridis* (Van der Heijden, et al., 2006). Three different genotypes were described: types I and II were associated with clinical disease and represented typical isolates of *H. meleagridis* at the morphological level whereas type III was not associated with clinical disease and morphologically slightly different from the types I and II. Moreover, ITS-1 sequences of type III showed numerous differences with those of the two other types and likely corresponded to a distinct species. However, although ITS-1 could be a valuable marker for genotypic approaches, the restricted number of positions used as well as the limited sampling of parabasalid species analysed represented restrictive factors for taxonomic considerations. Therefore, it was of interest to obtain the SSU rRNA gene sequences for each of the three genotypes described above that could be compared to those of a large panel of parabasalid species including *H. meleagridis*.

In this study, small subunit rRNA gene sequences were subsequently obtained by PCR from isolates belonging to the genotypes I, II, and III, and analysed in a broad molecular phylogeny of parabasalids. All these data allowed us to clarify

genetic diversity and species identities among isolates obtained from the caecum of fowl.

Materials and Methods

Source of trichomonad isolates and preparation of DNA. The isolation procedure and source of the three *H. meleagridis* isolates numbered 34, 17, and 23 analysed in this study as well as DNA extraction, were previously described in details (Van der Heijden et al., 2005, 2006). Briefly, isolates 34 and 17 originated from two flocks of turkeys and chickens, respectively, with acute histomonosis whereas isolate 23 originated from a flock of turkeys with no clinical histomonosis (Van der Heijden et al., 2006). Genotyping of these different isolates based on ITS-1 sequences revealed that isolates 34, 17 and 23 belonged to *H. meleagridis* types I, II, and III, respectively. DNA from these isolates was extracted using a commercial DNA isolation kit (Qiamp DNA minikit, Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Polymerase chain reaction amplification, cloning, and sequencing. Small subunit rRNA genes from isolates 34, 17, and 23, were amplified using the sense primer HistoF (5-AGGAAGCACACTATGGTCAT-3) and antisense primer HistoR (5-CGTTACCTTGTTACGACTTC-3). These primers were designed according to the published SSU rRNA gene sequence of *H. meleagridis* strain HmZL (Gerbod et al. 2001) and were complementary to the domains from positions 1 to 20 and 1582 to 1602, respectively (GenBank accesion No. AF293056). The PCR amplifications were carried out in 50 µl according to standard conditions for Platinum Taq DNA polymerase (Invitrogen, Groningen, The Netherlands). After the denaturation step at 94 °C for 5 min, 40 cycles of amplification were performed with a GeneAmp PCR System 9700 apparatus (Applied Biosystems, Courtaboeuf, France) as follows: 1 min at 94 °C, 1 min at 50 °C and 3 min at 72 °C. The final extension step was continued for 15 min. The products were separated by agarose gel electrophoresis, and the bands of the expected size (around 1,600 bp) were purified using the QIAEX II Gel Extraction Kit (Qiagen). Purified PCR products were cloned in the T-vector, pCR 2.1-TOPO (Invitrogen) and amplified in TOP10 competent cells. Minipreparations of plasmid DNA were done using the QIAprep Spin Miniprep Kit (Qiagen). Two clones containing inserts of approximately the expected size were sequenced for each *H. meleagridis* genotype. All genes were sequenced on both strands by primer walking using ABI dye-terminator chemistry. The SSU rRNA gene sequences obtained in this study have been deposited in GenBank under accession numbers EU647884 to EU647889.

Phylogenetic analyses. The SSU rRNA gene sequences obtained in this study were aligned using the ARB package (http://www.arb-home.de/) then refined manually by juxtaposing phylogenetically conserved secondary structures. These sequences were added to a large data set, including (i) 62 other identified parabasalid sequences representatives of the large diversity of this group and (ii) two previously published sequences from *H. meleagridis* (strains HmZL and TC6) retrieved from databases. Accession numbers for the sequences used in the phylogenetic analyses are indicated in Figure 2. We restricted the phylogenetic inferences to sites that could be unambiguously aligned (1,247 positions). Full-length alignment and boundaries are available upon request to the authors. Maximum likelihood analysis was carried out with PHYML (Guindon et al., 2005) using the general-time reversible model with gamma distributed rate variation and a proportion of invariable sites. The appropriate model of sequence evolution was selected using the program MODELTEST 3.06 (Posada and Crandall, 1998). Bootstrap values (BV) were obtained from 500 replicates. Bayesian analysis was also performed with MrBAYES 3_0b4 (Huelsenbeck and Ronquist 2001) for the same data set using the same model as described above and Bayesian posterior probabilities (BPP) were calculated. Starting tree was random and four simultaneous Markov chains were run for 500,000 generations with burn-in values set at 100,000 generations in order to ensure the use of only stable chains. All analyses were carried out excluding outgroups. Indeed, a major problem of rooting for the Parabasalia is the lack of any closed outgroup species. It is known that in-group relationships can change based on the choice of outgroup and this possibility cannot be excluded in parabasalid phylogenies. However, it has been shown that the relationships among and within parabasalid groups in rooted trees with various eukaryotic groups that did not significantly differ in their base composition from the parabasalid sequences, were generally in agreement with unrooted trees (Hampl et al., 2004).

Results and Discussion

Identification of trichomonad cells. In the caecal samples of birds analysed in this study, cells belonging to the types I and II isolates exhibited the same morphology (Figure 1) as previously described (Van der Heijden et al., 2006). These spherical cells measure about 15 µm in diameter, contain numerous vesicles and food vacuoles, and bear a single flagellum that is generally difficult to observe with phase contrast. Moreover, the ITS-1 sequences of the type I isolates were similar to those of the type II with few differences due to base substitutions and inserts or deletions of one or two bases (Van der Heijden et al., 2006). All these data strongly suggested that type I and type II isolates were likely derived from the same species *H. meleagridis* that is commonly found in the caecum of

birds. Interestingly, the ITS-1 sequences of the type I and type II isolates showed numerous differences with those of type III suggesting that the latter isolates were derived from a different species (Van der Heijden et al., 2006). At the morphological level, cells belonging to the type III are slightly larger in size (around 20 µm) than those of types I and II and have a more granular appearance (Figure 1). Moreover, it was not possible to subculture these non-pathogenic flagellates in Dwyer's medium or to re-start culture from cryopreserved isolates stored in liquid nitrogen in contrast to those of types I and II, preventing any ultrastructural study (Van der Heijden et al., 2006). In addition, the sequence of the 150 bp at the 3' end of SSU rRNA gene of type III isolates differed from those of *H. meleagridis*.

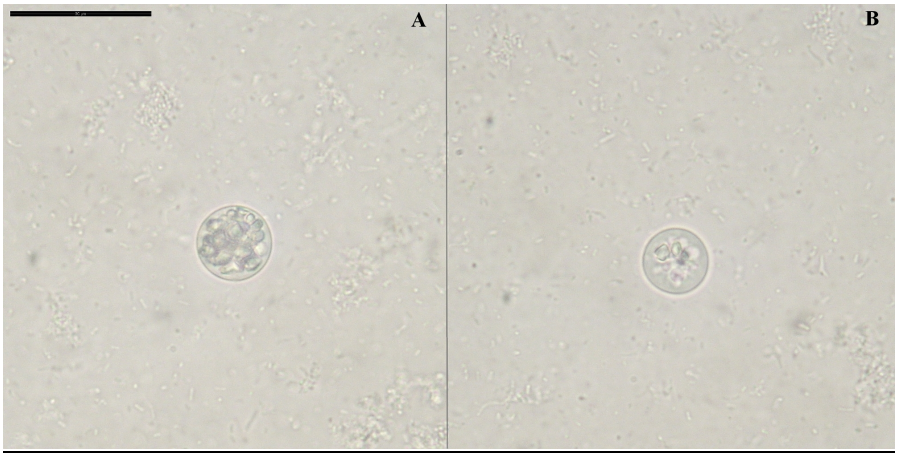


Figure 1: Phase-contrast micrographs of the trichomonads isolated from the caecum of fowl. A) Appearance of type III isolate 23 organisms corresponding to *P. wenrichi*. B) Cells belonging to type I isolate 34, exhibit a slightly different morphology with isolate 23 and were identified as *H. meleagridis*. Cells belonging to type II isolate 17, share the same morphology. Note that all the isolates are amoeboid and their flagella are not visible under microscopic examination. Reprinted by permission of the publisher Taylor and Francis Ltd from Van der Heijden et al. (2006). Bar = 30 µm.

However, the phylogenetic analysis of this short sequence showed that type III was closely related to *H. meleagridis* and *D. fragilis* and less related to other trichomonads (Van der Heijden et al., 2006). In particular, these microorganisms are fundamentally different at the morphological and molecular levels of the two other parabasalids that can be found in the caecum of birds, *Tetratrichomonas gallinarum* (Saleudin 1971) and *Trichomonas gallinae* (Mattern et al., 1967). Therefore, these observations strongly suggested that type III isolates could only correspond to *Parahistomonas wenrichi*. Indeed, all these data confirmed the morphological, culture, and pathogenic peculiarities of this microorganism

described in previous studies (Honigberg and Kuldova, 1969; Lund, 1963). However, *P. wenrichi* is characterized by the presence of four flagella and any flagellum was not clearly recognized in our type III isolates upon microscopic examination. It has been previously shown that in living organisms as well as in protargol staining of cell preparations, the flagella of *P. wenrichi* were extremely difficult to observe and numerous cells only exhibited one visible flagellum after staining (Honigberg and Kuldova, 1969). In addition, the cellular cycle of this trichomonad remains unknown and the existence of an aflagellated form during this cycle cannot be excluded as it has been described for *H. meleagridis* (Lund et al., 1967). Therefore, until further ultrastructural studies, type III isolates will be considered as belonging to *P. wenrichi*. However, the hypothesis that these isolates could represent a yet unidentified amoeboid species because of their morphological similarity with *H. meleagridis* cannot be excluded.

Phylogenetic analysis of small subunit rRNA gene sequences of bird isolates. In the aim to formally identify these trichomonads found in the caecum of birds, the SSU rRNA genes were amplified from the 3 isolates described above. The amplification produced a DNA fragment of the expected size (around 1,600 bp) as determined by gel electrophoresis. This fragment was eluted and cloned into the PCR 2.1-TOPO vector, and two clones were completely sequenced for each isolate. The sequences of the two clones of type I isolate 34 and type II isolate 17 exhibited only four and two differences, respectively. The dissimilarity observed between the sequences of these clones was likely due to the known micro-heterogeneity in the SSU rDNA coding region of parabasalids. Moreover, the sequences of these four clones showed only 11 variable positions between them (1,560 positions compared) indicating that all these clones were derived from the same species. About the two clones of type III isolate 23, they showed 28 differences (1,625 positions compared). This level of difference could also be explained by the polymorphism of the SSU rRNA genes.

The SSU rRNA gene sequences obtained in this study were added to an existing database of 64 other parabasalid sequences including in particular those of the *H. meleagridis* strains ZmHL and TC6. The unrooted trees reconstructed in this study (Figure 2) identified four clades mostly well supported by BV/BPP values that were consistent with those described in previous phylogenetic studies (Dufernez et al., 2007; Gerbod et al., 2002; Hampl et al., 2004, 2006, 2007; Keeling, 2002; Noël et al., 2007; Ohkuma et al., 2005). Briefly, clade 1 corresponds to the order Trichonymphida. Clade 2 and 4 consist of the orders Spirotrichonymphida and Cristamonadida, respectively, whereas clade 3 is represented by the very heterogeneous and paraphyletic / polyphyletic order Trichomonadida that includes almost all the Monocercomonadidae such as *Histomonas*. In addition to these clades, we also identified a discrete lineage represented by the only one Monocercomonadidae *Hexamastix*.

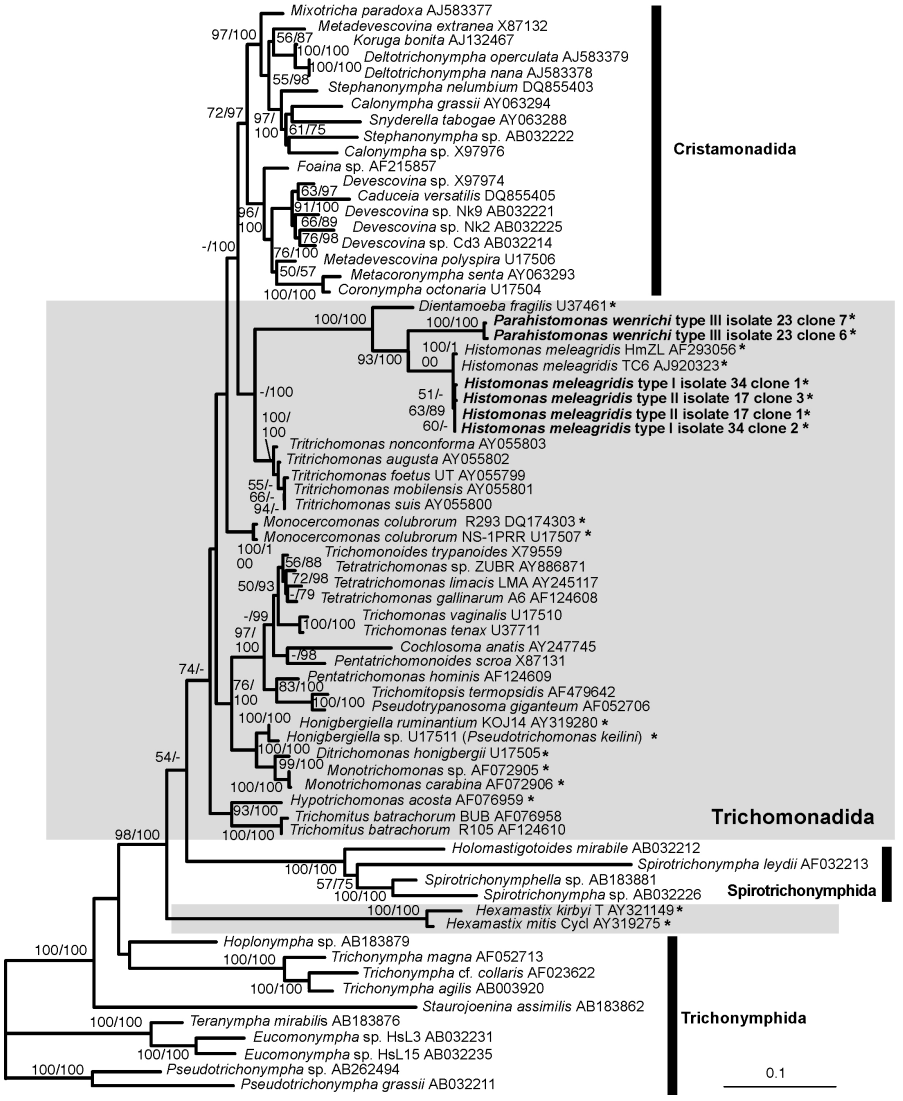


Figure 2: Maximum likelihood tree of parabasalids based on SSU rRNA gene sequences. The trichomonad sequences obtained in this study are indicated in bold. Accession numbers of the sequences used in the analysis are indicated. Numbers near the individual nodes indicate bootstrap values (left of the slash) and Bayesian posterior probabilities (right of the slash) given as percentages by the two different reconstruction methods (maximum likelihood/MrBAYES). Nodes with values below 50% are not shown. Parabasalid orders as in Adl et al. (2005) are bracketed to the right or indicated by shaded box. Note paraphyly / polyphyly of the order Trichomonadida. Representatives of the Monocercomonadidae family are indicated by stars. Scale bar indicates 0.1 substitutions (corrected) per base pair.

Concerning isolates 34 and 17 corresponding to the types I and II, respectively, and both identified as *H. meleagridis* on the base of various peculiarities including morphological criteria, our phylogenetic analyses confirmed the identification of these flagellates as *H. meleagridis*. Indeed, these isolates and *H. meleagridis* strains HmZl and TC6 grouped together with BV/BPP of 100%/100%. The SSU rRNA gene sequences obtained from isolates 34 and 17 shared high sequence similarity (99.1 to 99.8% identity and 1,560 positions compared) with those of the *H. meleagridis* strains HmZl and TC6. These data indicate that the trichomonads examined in these two isolates are closely related, if not identical, to *H. meleagridis*.

The SSU rRNA gene sequences of the two clones of type III isolate 23, cluster together with BV/BPP of 100%/100%. The evolutionary distances observed between types III and I/II isolates are greater than those calculated between most of the other trichomonad genera (Figure 2). Indeed, the sequences of the two clones of type III exhibit only 83.7 to 84.3% identity (1,630 positions compared) with those of types I and II. Therefore, type III isolates cannot be derived from *H. meleagridis* and these data confirm their likely identification as *P. wenrichi*. Interestingly, *P. wenrichi* shows a strong sister-group relationship with *H. meleagridis* strains (BV/BPP of 93%/100%) suggesting a common evolutionary origin. *Parahistomonas* and *Histomonas* group consistently with *D. fragilis* with BV/BPP of 100%/100% and the large group composed by these three genera share a common ancestor with *Tritrichomonas*. This clustering with *Tritrichomonas* is strongly supported by BPP of 100% but not by BV and confirms previous phylogenetic inferences suggesting the grouping of *Tritrichomonas*, *Dientamoeba*, and *Histomonas* (Dufernez et al., 2007; Gerbod et al., 2001; Keeling, 2002; Noël et al., 2007; Ohkuma et al., 2005). By showing *Histomonas* and *Dientamoeba* to be derived from forms with complex cytoskeletal development such as *Tritrichomonas*, it reinforces the hypothesis that the relative morphological simplicity of these two amoeboid genera is linked to the secondary reduction or loss of some cytoskeletal structures as previously suggested (Gerbod et al., 2001). According to our phylogenetic tree, we observe a reduction of the number of flagella in *Histomonas* (only one flagellum) and *Dientamoeba* (no flagellum) in the course of evolution in comparison with the four flagella described in *Tritrichomonas* (Honigberg et al., 1971) while this reduction should not occur in *Parahistomonas* (four flagella). On the contrary, the enhancement of amoeboid tendency and reduction in the size of the axostyle-pelta complex is clearly confirmed in *Parahistomonas* by our molecular data as observed for *Dientamoeba* and *Histomonas*. Since *Parahistomonas* is considered to be non-pathogenic, these observations indicate that the secondary loss of cytoskeletal structures is not always correlated with a parasitic mode of life as it has been previously proposed for the free-living Monocercomonadidae (Edgcomb et al. 1998). Moreover, as stated above, *Histomonas* and *Parahistomonas* share a common ancestor according to our

phylogenetic tree and are both found in the caecum of galliform birds including chickens, turkeys, and pheasants. Since they are i) colonizing the same hosts and environmental niche and ii) phylogenetically and morphologically related, we consider that it is more likely that speciation leading to these two taxa recently occurs in birds already infected by the ancestor of these two trichomonad genera. Indeed, two independent colonizations of the caecum of birds by *Histomonas* and *Parahistomonas* in the course of evolution remain hypothetical according to their close relationships. In addition, our data do not allow us to determine if the ancestor of these two taxa was pathogenic or not. It means that either *Parahistomonas* could be primarily non-pathogenic as its ancestor or it has lost its ancestral pathogenicity which was retained in *Histomonas*. However, since *Parahistomonas* is the sole non-pathogenic species in the well-resolved clade grouping together this genus with *Tritrichomonas*, *Dientamoeba*, and *Histomonas*, the hypothesis of a pathogenic ancestor of *Parahistomonas* remains the more probable and by consequence, a secondarily loss of its pathogenicity. Whatever could be the ancestral mode of life of these two trichomonads, we have identified an outstanding example of parallel adaptation of two organisms to the same host following speciation through the study of *Histomonas* and *Parahistomonas*. Finally, to continue with our taxonomic considerations, one is struck by the great morphological similarity which seems to exist between *P. wenrichi* and *Protrichomonas legeri*, a trichomonad living in most cases in the oesophagus of *Box boops* but also reported in ducks (Brugerolle, 1980). Indeed, *P. legeri* also possesses 4 flagella (3 anterior flagella and an intracytoplasmic recurrent flagellum), a similar size to that of *P. wenrichi*, and an amoeboid tendency. Thus, Honigberg and Kuldova (1969) suggested that these two species would have to be considered as congeneric, with *Protrichomonas* as the type genus. In further studies, it would be of great interest to obtain the SSU rRNA gene sequence of *P. legeri* that could be now compared to that of *P. wenrichi* in order to test the proposed synonymy of *Parahistomonas* and *Protrichomonas*.

In conclusion, the molecular identification of a trichomonad species described as *P. wenrichi* in fowl is the striking point of our study. Indeed, until now, its existence was proposed on the only base of few observations and its phylogenetic relationships with other parabasalid species remained unknown. Moreover, its common ecological niche and morphological similarity with *H. meleagridis* may confuse the diagnosis of histomonosis. Indeed, it remains extremely difficult not to say impossible for the observer to distinguish between *Parahistomonas* and *Histomonas* with certainty by microscopic examination (Lund 1963). Thus, the potential presence of *Parahistomonas* cells in the collected bird samples has to be taken in account by poultry specialists since these *Histomonas*-like microorganisms could lead to a false-positive diagnosis of histomonosis in some clinical contexts.

Acknowledgements

This work was supported by the Institut Pasteur de Lille, the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, and the Université de Lille 2 to E. V., Ecomolecular Science Research Program and Bioarchitect Research Program grants from RIKEN to M. O., and by a grant from the French Ministère de l'Education Nationale, de la Recherche et de la Technologie to C. M.

References

Adl, S.M., Simpson, A.G.B., Farmer, M.A., Andersen, R.A., Anderson, O.R., Barta, J.R., Bowser, S.S., Brugerolle, G., Fensome, R.A., Fredericq, S., James, T.Y., Karpov, S., Kugrens, P., Krug, J., Lane, C.E., Lewis, L.A., Lodge, J., Lynn, D.H., Mann, D.G., McCourt, R.M., Mendoza, L., Moestrup, O., Mozley-Standridge, S.E., Nerad, T.A., Shearer, C.A., Smirnov, A.V., Spiegel, F.W., Taylor, M.F.J.R., 2005, The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. Journal of Eukaryotic Microbiology 52, 399-451.

Brugerolle, G., 1976, Cytologie ultrastructurale, systématique et évolution des Trichomonadida. Annales de la Station Biologique de Besse-en-Chandesse 10, 1-90.

Brugerolle, G., 1980, Etude ultrastructurale du flagellé *Protrichomonas legeri* (Léger 1905) parasite de l'estomac des Bogues (*Box boops*). Protistologica 17, 431-438.

Brugerolle, G., Lee, J.J., 2001, Phylum Parabasalia. In: Lee, J. J., Leedale, G. F., Patterson, D. J. & Bradbury, P. C. (ed.), The Illustrated Guide to the Protozoa. 2nd ed. Allen Press, Lawrence, Kansas, pp. 1196-1249.

Carpenter, K.J., Keeling, P.J., 2007, Morphology and phylogenetic position of *Eucomonympha imla* (Parabasalia: Hypermastigida). Journal of Eukaryotic Microbiology 54, 325-332.

Dufernez, F., Walker, R.L., Noël, C., Caby, S., Mantini, C., Delgado-Viscogliosi, P., Ohkuma, M., Kudo, T., Capron, M., Pierce, R.J., Villanueva, M.R., Viscogliosi, E., 2007, Morphological and molecular identification of non-*Tritrichomonas foetus* trichomonad protozoa from the bovine preputial cavity. Journal of Eukaryotic Microbiology 54, 161-168.

Edgcomb, V.P., Viscogliosi, E., Simpson, A.G.B., Delgado-Viscogliosi, P., Roger, A.J., Sogin, M.L., 1998. New insights into the phylogeny of trichomonads inferred from small subunit rRNA sequences. Protist 149, 359-366.

Gerbod, D., Edgcomb, V.P., Noël, C., Zenner, L., Wintjens, L., Delgado-Viscogliosi, P., Holder, M.E., Sogin, M.L., Viscogliosi, E., 2001, Phylogenetic position of the trichomonad parasite of turkeys, *Histomonas meleagridis* (Smith) Tyzzer, inferred from small subunit rRNA sequence. Journal of Eukaryotic Microbiology 48, 498-504.

Gerbod, D., Noël, C., Dolan, M.F., Edgcomb, V.P., Kitade, O., Noda, S., Dufernez, F., Ohkuma, M., Kudo, T., Capron, M., Sogin, M.L., Viscogliosi, E., 2002, Molecular phylogeny of parabasalids inferred from small subunit rRNA sequences, with emphasis on the Devescovinidae and Calonymphidae (Trichomonadea). Molecular Phylogenetics and Evolution 25, 545-556.

Guindon, S., Lethiec, F., Duroux, P., Gascuel, O., 2005, PHYML online – a web server for fast maximum likelihood-based phylogenetic inference. Nucleic Acids Research 33, W557-W559.

Hampl, V., Cepicka, I., Flegr, J., Tachezy, J., Kulda, J., 2004, Critical analysis of the topology and rooting of the parabasalian 16S rRNA tree. Molecular Phylogenetics and Evolution 32, 711-723.

Hampl, V., Vrlik, M., Cepicka, I., Pecka, Z., Kulda, J., Tachezy, J., 2006, Affiliation of *Cochlosoma* to trichomonads confirmed by phylogenetic analysis of the small-subunit rRNA gene and a new family concept of the order Trichomonadida. International Journal of Systematic and Evolutionary Microbiology 56, 305-312.

Hampl, V., Cepicka, I., Flegr, J., Tachezy, J., Kulda, J., 2007, Morphological and molecular diversity of the monocercomonadid genera Monocercomonas, Hexamastix, and Honigbergiella gen. nov. Protist 158, 365-383.

Honigberg, B.M., 1963, Evolutionary and systematic relationships in the flagellate order Trichomonadida Kirby. Journal of Protozoology 10, 20-63.

Honigberg, B.M., Brugerolle, G., 1990, Structure. In: Honigberg, B. M. (ed.), Trichomonads Parasitic in Humans. Springer-Verlag, New-York. pp. 5-35.

Honigberg, B.M., Kuldova, J., 1969, Structure of a nonpathogenic histomonad from the cecum of galliform birds and revision of the trichomonad family Monocercomonadidae Kirby. Journal of Protozoology 16, 526-535.

Honigberg, B.M., Mattern, C.F.T., Daniel, W.A., 1971, Fine structure of the mastigont system in *Tritrichomonas foetus* (Riedmüller). Journal of Protozoology 18, 183-198.

Huelsenbeck, J.P., Ronquist, F., 2001, MrBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754-755.

Keeling, P.J., 2002, Molecular phylogenetic position of *Trichomitopsis termopsidis* (Parabasalia) and evidence for the Trichomitopsiinae. European Journal of Protistology 38, 279-286.

Lund, E.E., 1963, *Histomonas wenrichi* n. sp. (Mastigophora: Mastigamoebidae), a non-pathogenic parasite of gallinaceous birds. Journal of Protozoology 10, 401-404.

Lund, E.E., 1969, Histomoniasis. Advances in Veterinary Science and Comparative Medicine 13, 355-390.

Lund, E.E., Augustine, P.C., Chute, A.M., 1967, *Histomonas meleagridis* after one thousand *in vitro* passages. Journal of Protozoology 14, 349-351.

Mattern, C.F.T., Honigberg, B.M., Daniel, W.A., 1967, The mastigont system of *Trichomonas gallinae* (Rivolta) as revealed by electron microscopy. Journal of Protozoology 14, 320-339.

McDougald, L.R. 1997. Protozoa: other protozoan diseases of the intestinal tract. In: Calneck, B., Barnes, H. J., Beard, C. W., McDougald, L. R. & Saif, Y. M. (ed.), Diseases of Poultry, 10th edn. Ames: Iowa State University Press. pp. 890-899.

Noël, C., Noda, S., Mantini, C., Dolan, M.F., Moriya, S., Delgado-Viscogliosi, P., Kudo, T., Capron, M., Pierce, R.J., Ohkuma, M., Viscogliosi, E., 2007, Molecular phylogenetic position of the genera *Stephanonympha* and *Caduceia* (Parabasalia) inferred from nuclear small subunit rRNA gene sequences. Journal of Eukaryotic Microbiology 54, 93-99.

Ohkuma, M., Iida, T., Ohtoko, K., Yuzawa, H., Noda, S., Viscogliosi, E., Kudo, T., 2005, Molecular phylogeny of parabasalids inferred from small subunit rRNA sequences, with emphasis on the Hypermastigea. Molecular Phylogenetics and Evolution 35, 646-655.

Posada, D., Crandall, K.A., 1998, MODELTEST: testing the model of DNA substitution. Bioinformatics 14, 817-818.

Reid, W.M., 1967, Etiology and dissemination of the blackhead disease syndrome in turkeys and chickens. Experimental Parasitology 21, 249-275.

Rybicka, K., Honigberg, B.M., Holt, S.C., 1972, Fine structure of the mastigont system in culture forms of *Histomonas meleagridis* (Smith). Protistologica 8, 107-120.

Saleudin, A.S.M., 1971, Fine structure of *Tetratrichomonas limacis* (Dujardin). Canadian Journal of Zoology 50, 695-701.

Schuster, F.L., 1968., Ultrastructure of *Histomonas meleagridis* (Smith) Tyzzer, a parasitic ameboflagellate. Journal of Parasitology 54, 725-737.

Smith, T., 1895, An infectious disease among turkeys caused by protozoa (infectious entero-hepatitis). Bulletin of the United States Department of Agriculture 8, 7-38.

Tyzzer, E.E., 1920, The flagellate character and reclassification of the parasite producing “blackhead” in turkeys – *Histomonas* (gen. nov.) *meleagridis* (Smith). Journal of Parasitology 6, 124-131.

Tyzzer, E.E., 1934, Studies of histomoniasis, or "blackhead" infection, in the chickens and the turkey. Proceedings of the American Academy of Arts and Sciences 69, 189-264.

Van der Heijden, H.M.J.F., McDougald, L.R., Landman, W.J.M., 2005, High field of parasites and prolonged *in vitro* culture of *Histomonas meleagridis*. Avian Pathology 34, 505-508.

Van der Heijden, H.M.J.F., Landman, W.J.M., Greve, S., Peek, R., 2006, Genotyping of *Histomonas meleagridis* isolates based on internal transcribed spacer-1 sequences. Avian Pathology 35, 330-334.

Wenrich, D.H., 1943, Observations on the morphology of *Histomonas* (Protozoa, Mastigophora) from pheasants and chickens. Journal of Morphology 72, 279-304.

Chapter 6

Summarizing discussion

Introduction

The aim of the studies described in this thesis was to optimize and develop new methods for the detection and typing of *Histomonas meleagridis* and to examine new possibilities to control histomonosis in commercial poultry. The studies focussed on the improvement of *H. meleagridis* culture, resulting in a less complex medium and a higher yield of parasites (Chapter 2), the development of a blocking-ELISA for detection of antibodies (Chapter 4) and a C-profiling technique for genotyping (Chapter 5) as new laboratory tools, and examination of possible antihistomonal compounds using *in vitro* and *in vivo* models (Chapter 3).

In this summarizing discussion, the field validation and possible applications of the new laboratory tools are discussed (Enzyme-Linked ImmunoSorbent Assay and Molecular techniques). Further, a number of candidate antihistomonal products for control of histomonosis as well as the in *vitro* and *in vivo* models to screen them are considered (Antihistomonals). It is contended (*Dientamoeba fragilis)* that research institutes investigating either the human pathogen *Dientamoeba fragilis* or the veterinary pathogen *H. meleagridis* should cooperate. Since there possibly is a missing link in the epidemiology of histomonosis, the last paragraph is dedicated to transmission routes and vectors (Transmission). This summarizing discussion concludes with suggestions for future research.

Antihistomonals

Following the ban on effective chemotherapeutics there was an urgent quest for new and safe antihistomonals to control histomonosis. The most cost-effective strategy to examine candidate products is to use *in vitro* testing for screening and confirm the antihistomonal properties found in an *in vivo* study. Pre-screening also reduces the need for animal experiments, although products that only have an antihistomonal activity after metabolization in the host will be missed. Before being able to perform *in vitro* experiments, it was necessary to develop a reproducible culture method. In Chapter 2 of this thesis, considerable improvements to the commonly used medium for culturing *H. meleagridis* are presented. In the original description of Dwyer's medium (Dwyer, 1970) it was stated that the amount of rice starch in the culture medium was critical with 10 to 12 mg per 10 ml of culture medium without providing experimental evidence. In Chapter 2 it is shown that the parasite yield was approximately 10 times higher when a larger amount of 50 to 100 mg of rice powder was used instead. The size of the rice particles added proved relatively unimportant. Another conclusion from the studies performed was that only by addition of rice the culture's life could be prolonged, making subculturing every two or three days less necessary thus making maintenance of the cultures easier. This finding was in agreement with observations reported by

Tyzzer without him providing experimental results (Tyzzer, 1934). Alternative sources of starch have been reported as suitable culture medium components (McDougald, 2005), but were not examined here. A further improvement of the culture medium described in Chapter 2 was the observation that chicken embryo extract (CEE), which is difficult to standardize, was redundant. Another group recently reported that histomonads also grow well when CEE was omitted from Stepkowski-Klimont medium (Hess et al., 2006), which is similar to Dwyer's medium. The other main medium constituent, horse serum, however, proved indispensable (Chapter 2). There is some controversy about the dependence of histomonads on cecal bacteria during culturing (McDougald, 2005). In our hands, elimination of the culture bacteria by addition of gentamycin resulted in non-viable histomonad cultures (unpublished data). Another observation made during our studies was that cultures of 12.5 ml in 70 ml tissue culture flasks performed better than cultures in larger volumes of medium or smaller tubes (unpublished data). Possibly, histomonads or part of the accompanying bacterial flora are not strictly anaerobic and a delicate balance exists between the volumes of air and culture medium in the culture flask. It remains to be investigated, however, whether strict anaerobic conditions would represent a further improvement. Perhaps that could also be the case for *Parahistomonas wenrichi*, which cannot be cultured using standard media and conditions (Honigberg and Bennett, 1971).

Improved cultures with higher yields of histomonads may be useful for the production of antigen for ELISA or vaccines. Recently, it was shown that vaccination with a prototype live attenuated vaccine of a clonal histomonad culture protected turkeys against histomonosis (Hess et al., 2006 and 2008), despite disappointing results reported in the past (Lund, 1959; Tyzzer, 1936). Apart from research into the immune response elicited by vaccination (Hess et al., 2008) many other topics like upscaling of culture, stability of histomonads, strain differences, routes of application and safety need to be investigated before field application is feasible.

From pilot experiments performed to develop an *in vivo* model (unpublished data), we learnt that the inoculation procedure is critical. If after intracloacal inoculation the birds were returned to their cages without taking precautions, they often defecated and as a consequence lost the inoculum. If before inoculation individual birds were placed in a cardboard box and allowed to defecate, a finger was placed on the cloaca for 30 seconds immediately following inoculation, and the birds were subsequently kept in inverted position for some minutes, this rarely occurred. If an accurate inoculation procedure ensures that (almost) all birds become infected after inoculation, a smaller number of birds per experimental group could be used without loss of statistical power.

Using *in vitro* and *in vivo* models a number of compounds and natural products were tested for antihistomonal efficacy in Chapter 3. Some natural products had been marketed as feed additives with a possible antihistomonal effect. As turkey

producers no longer had effective chemotherapeutics at their disposal, they were tempted to buy these feed additives although scientific evidence for their antihistomonal effect was lacking. Despite the fact that two herbal products, Enteroguard™ and Protophyt™, were found to have an inhibitory effect on histomonal growth *in vitro*, (Chapter 3.1) they had no protective effect in turkeys intracloacally challenged with *H. meleagridis* (Chapter 3.2). The antihistomonal *in vitro* effect may have been caused by a bactericidal effect of the herbal products on one or more cecal bacterial strains that *H. meleagridis* is dependent on during culturing. Another explanation could be that the products did not accumulate to a sufficiently high level in the cecum of the birds. This illustrates the necessity for *in vivo* studies to confirm an antihistomonal *in vitro* effect. For Protophyt™ contradicting results have been reported. This product was ineffective in a field study (Chossat, 2002) but, in contrast to our results (Chapter 3.2), a statistically significant antihistomonal effect was observed during an *in vivo* experiment (Hafez and Hauck, 2006). Instead of the "standard" inoculation dose of 200,000 histomonads per bird, they used a slightly lower inoculation dose of 147,500 histomonads per bird. But the difference in findings between the *in vivo* studies could not be attributed to a dose effect (Lund, 1955) as in our experiment in addition to the "standard" inoculation dose, a much lower inoculation dose (3,162 histomonads per bird) was employed and resulted in histomonosis in the positive control group (no treatment) and the experimental groups receiving feed additives. The difference in the observed results for Protophyt™ may have been caused by different virulences of the strains used. The establishment of an international reference strain to be included as a control in addition to experiments with isolates from the target population, would favour standardization and enable better comparison between experimental studies. A *H. meleagridis* strain that was cloned from a single cell following micromanipulation (Hess et al., 2006) is probably the best defined isolate (Grabensteiner et al., 2007; Grabensteiner et al., 2006; Hess et al., 2006; Hess et al., 2008; Liebhart et al., 2008; Liebhart et al., 2006; Singh et al., 2008) at the time and could be suited as the international reference strain.

Other recent studies have also identified natural products like coated plant extracts (Hauck and Hafez, 2007), carvacrol and a saponin (Grabensteiner et al., 2007) as possible antihistomonals after *in vitro* analysis. However, their antihistomonal *in vivo* activity has not been assessed yet.

In Chapter 3.3 two antibiotics, tiamulin and paromomycin, were tested for antihistomonal effect. Tiamulin had been applied in a field case (Burch et al., 2007), but since untreated controls were missing it was uncertain whether the observed decline in mortality could be attributed to the antibiotic treatment or was merely coincidental. No effect of tiamulin was found in our *in vitro* model. In contrast, a clear antihistomonal effect was found for paromomycin, both *in vitro* and *in vivo*. After its administration as a feed additive, a clear dose response effect was observed, with no protection at 100 ppm, partly protection, especially in hens, at

200 ppm and full protection against an otherwise lethal challenge at 400 ppm. Paromomycin is an aminoglycoside antibiotic derived from *Streptomyces chrestomyceticus*. Its mechanism of action in susceptible prokaryotes and protozoa is based on binding to decoding regions of ribosomal RNA, interfering with protein synthesis (Gardner and Hill, 2001; Kotra et al., 2000; Marouf et al., 1995; Schroeder et al., 2000). It was also reported to be effective against *D. fragilis* (Chan et al., 1994; Vandenberg et al., 2007), which is closely related to *H. meleagridis*. Antihistomonal activity of paromomycin was reported more than 45 years ago (Lindquist, 1962), resulting in partly protection of turkeys against histomonosis in turkeys when supplied in the feed at very high concentrations (1000 and 2000 ppm) 4 to 8 days before inoculation with heterakids ova infected with *H. meleagridis*. In a more recent study in chickens, paromomycin had no protection when given at concentrations of 200 and 400 ppm in feed one day before inoculation with histomonads (Hu and McDougald, 2004). Recently, a dose related antihistomonal effect of paromomycin in turkeys was found when it was supplied in concentrations between 25 and 100 ppm in drinking water 3 days before infection (Hafez et al, 2008). There are many differences between these experiments (species of birds, inoculation routes, concentrations, timing of medication), therefore further investigations are necessary to pinpoint the factors causing the contradicting results. Also, so far only the preventive antihistomonal effect of paromomycin has been assessed. The next step is to examine whether paromomycin also has a curative antihistomonal effect. Under practical conditions curative treatment of histomonosis during outbreaks is preferable over preventive treatment. Major drawbacks for preventive use are costs and development of resistance. Paromomycin is similar to other aminoglycosides like neomycin, kanamycin, framycetin and streptomycin with cross-resistance between aminoglycosides occurring (Shaw et al, 1993). Continuous preventive antihistomonal treatment of turkeys with paromomycin may therefore result in resistance against antibiotics needed for treatment of bacterial diseases.

Since the ban on effective antihistomonal therapeutics, turkey owners no longer had a product at their disposal to treat histomonosis. Paromomycin has a Maximum Residue Limit (MRL) for meat producing animals and is registered for use in chickens to treat colibacillosis and salmonellosis. Since no antihistomonal product is available, the cascade system allows off-label use of paromomycin for treatment of histomonosis. This is especially useful for farmers having suffered from a large outbreak and whom are at risk to face the same problem in the next round. A practical solution for them might be preventive chemotherapy of the susceptible young turkeys by addition of 400 ppm to the feed until 17 weeks of age.

As *Heterakis gallinarum* is the most important vector for *H. meleagridis*, it has been suggested that antihelminth treatment is another option to control histomonosis in turkey and chicken flocks. Benzimidazole derivatives are cholinesterase inhibitors known to be effective against nematodes (Reuvekamp et

al., 2008). The two benzimidazole derivates albendazole and fenbendazole proved non-effective as curative antihistomonal treatment (during 5 days), but were effective when applied as prophylactic treatment (during 14 days). In these experiments turkeys were placed on broiler breeder litter contaminated with *H. gallinarum* ova containing *H. meleagridis* (Hegngi et al., 1999). The only benzimidazole derivate that is permitted in the Netherlands, flubendazole, has not been examined for its indirect antihistomonal effect. However, it is not likely that worm medication is very useful in preventing histomonosis in turkey flocks. Antihelminth treatment with flubendazole lasts 7 days and is repeated at intervals of several weeks. Continuous treatment is not possible because of development of resistance. Even if an antihelminth treatment would be 100% successful, which is unlikely (Reuvekamp et al., 2008), it will not prevent the introduction of worm eggs at a later point in time. Also since *H. meleagridis* is able to quickly spread within a turkey flock through cloacal drinking, i.e. independent of *H. gallinarum*, preventive antihelminth treatment is not likely to be of great value in control of histomonosis.

Enzyme-linked ImmunoSorbent Assay

In Chapter 4 the development of a blocking-ELISA for the serological detection of *H. meleagridis* infection is described. Validation of the assay was done using experimental sera. Chicks and poults quickly seroconverted after experimental inoculation with *H. meleagridis*. The test did not suffer from crossreactivity with the closely related avian protozoan *Tetratrichomonas gallinarum*, because the monoclonal conjugate did not bind to *T. gallinarum* antigen. Moreover, layers inoculated with *T. gallinarum* did not respond in the *H. meleagridis* blocking-ELISA, while intramuscularly inoculated birds did seroconvert against *T. gallinarum*. Since diagnosis of histomonosis in turkeys is easily performed using gross pathology and sometimes histology as confirmation, a blocking-ELISA will be of limited added value as a diagnostic tool for detection clinical outbreaks of histomonosis in turkey flocks. However, it may prove very valuable for research purposes. The assay could be applied in experimental transmission studies where only a proportion of the birds is inoculated as a more sensitive technique to detect (protection against) transmission of histomonads between turkeys or chickens. So far, in such experiments only morbidity, mortality, weight gain and lesion scores were available for comparisons between groups. Despite the fact that the immune response against *H. meleagridis* is probably based on cellular immunity rather than the humoral response (Hess et al., 2008), another research application of the ELISA could be to evaluate humoral responses of vaccination during the development of vaccines, e.g. comparison of vaccine strains, timing, routes and schemes.

Further, the blocking-ELISA could be used to perform seroepidemiological studies especially in chicken farms. Chickens may act as a reservoir of the

parasite, but it is unknown what proportion of the flocks is infected. A serological survey (30 serum samples per flock) on different types of Dutch layer farms (12 organic flocks, 24 free-ranging flocks, 39 flocks with floor housing, and 40 flocks with cage housing) with a median age of 492 days showed 15 to 18% strong positive results not relating to the type of flock (unpublished data). It remains to be investigated whether the positive flocks were indeed infected, or that the results were caused by crossreactivity with a widely distributed antigen or micro-organism other than *T. gallinarum*. The former explanation is the most likely, as *H. gallinarum*, the vector of *H. meleagridis*, is common in chickens. In 2003 *H. gallinarum* infection was found (Reuvekamp et al., 2008) in 8.9% of necropsies of chickens performed at the Dutch Animal Health Service, while in the USA most broiler breeder pullet farms have found to be contaminated with cecal worm eggs (McDougald, 2005).

Molecular techniques

In Chapter 5 a novel molecular typing technique is described for genotyping *H. meleagridis* isolates. It is based on PCR and sequencing of the Internal Transcribed Spacer 1 (ITS1) region associated with the rRNA genes which are present in multiple copies per cell. Unlike the rRNA genes, this region is sufficiently variable to enable genotyping. The ITS1 region has a very low cytosine (C) content. When the A, T and G patterns were omitted from the otherwise uninterpretable sequencing chromatograms, a highly reproducible C-profile was obtained. This typing technique was therefore called C-profiling.

Initially three subtypes (C-profiles) were distinguished, of which type III has been further characterized as being closely related to *H. meleagridis*, but belonging to a different genus, possibly *P. wenrichi*. C-profiling could prove a useful tool for epidemiological studies by comparing *H. meleagridis* from different sources. But it would be especially useful if different *H. meleagridis* genotypes were linked to relevant phenotypes (virulence). This is an area of research that deserves further attention.

Dientamoeba fragilis

H. meleagridis is closely related to the human pathogen *D. fragilis*, which may cause abdominal pain, diarrhoea, vomiting and fatigue (Johnson et al., 2004). Differently from the former it has two nuclei and no flagellum. The protozoa are antigenically (Dwyer, 1972a, b, 1974) and genetically (Gerbod et al., 2001) related. Other than the histomonas flagellum, which is not easy to see at light microscopy, and the two nuclei of *D. fragilis*, the parasites have similar morphology, ingest rice

particles when cultured, and are easily cultured *in vitro* with more or less comparable media. The transmission route of both parasites has not been fully elucidated yet. The cecal worm *H. gallinarum* is the key vector in the life cycle of *H. meleagridis* (Graybill and Smith, 1920). In the case of *D. fragilis* the pinworm *Enterobius vermicularis* is assumed to act as an intermediate vector (Johnson et al., 2004). In birds, the cecum is the primary target for *H. meleagridis*, while in humans appendices obtained in surgery often contain *D. fragilis* (Johnson et al., 2004). Speculations about the existence of a cyst stage for both parasites were published recently (Johnson et al., 2004; Mielewczik et al., 2008), although convincing scientific evidence is lacking.

Despite many similarities between both parasites and the fact that some of them were already observed more than half a century ago (Dobell, 1940), research has largely been independent. Human and veterinary research could benefit here from cooperation, e.g. the culture medium that was optimized and improved for *H. meleagridis* (Chapter 2) could prove equally useful for *D. fragilis*. This would facilitate the production of antigen to develop a (subtype) specific serological test like ELISA, which is currently not available for the human pathogen (Johnson et al., 2004). A major problem in *D. fragilis* research is that an adequate animal model is lacking (Johnson et al., 2004). Possibly, the well-established *H. meleagridis* model in turkeys could be useful in *D. fragilis* research. Given the close similarity of the parasites, candidate compounds for which an antihistomonal *in vivo* activity is found, may also show efficacy against *D. fragilis*. An example of a fruitful cooperation between human and veterinary research is the development of a genotyping technique described in Chapter 5 of this thesis.

Transmission

It is by no means certain that the vector *H. gallinarum* (Graybill and Smith, 1920) and “cloacal drinking” (Hu and McDougald, 2003) are the only routes of transmission of *H. meleagridis* from one flock to the other, between species of birds, or directly from bird to bird. In recent years, outbreaks of histomonosis in French turkey breeder flocks with high biosecurity were more frequent than in commercial meat turkeys (Callait-Cardinal et al., 2007; Leveque et al., 2007). In some of these flocks no evidence of *H. gallinarum* was found (Chossat, 2002) and the infection was therefore attributed to another unknown transmission route.

A limited number of small but nevertheless interesting studies on the possible role of invertebrates as mechanical carriers of cecal worm eggs have been performed (Frank, 1953; Tyzzer, 1920). Some species of flies were identified as mechanical carriers of cecal worm eggs (Frank, 1953) and it even proved possible to initiate histomonosis in turkeys by feeding them with grasshoppers that had been fed on lettuce sprinkled with *H. gallinarum* ova (Frank, 1953). Modern techniques

like PCR (Bleyen et al., 2007; Grabensteiner and Hess, 2006; Hafez et al., 2005; Huber et al., 2005; Stark et al., 2006) and optimized culture (Chapter 2) are now available to study the role of invertebrates in the mechanical transmission of *H. meleagridis* in greater depth.

So far invertebrates only were considered to be mechanical carriers of infected cecal worm eggs (McDougald, 2005). The possibility that they may act as intermediate or paratenic hosts was not studied until recently. The lesser mealworm *Alphitobius diaperinus*, which is often present in poultry houses, was investigated using PCR for histomonal DNA and culture for viability of *H. meleagridis* (Huber et al., 2007). In a small proportion of larvae from flocks suffering from histomonosis and in artificially infected insects, viable histomonads were detected. However, all adult beetles tested were negative by both PCR and culture. The authors therefore concluded that *A. diaperinus* is probably not of major importance in the transmission of *H. meleagridis* between flocks as under normal circumstances only adults are likely to move from flock to flock. Although the PCR was very sensitive and was reported to have a detection limit of 1 and 10 histomonads per adult beetle, or larvae, respectively, it is unfortunate that no *in vivo* studies were performed by feeding the adult insects to turkeys.

Some insects like termites are known to harbour many protozoa, including several parabasalids, in their posterior gut (Gerbod et al., 2000). Some of these protozoa are endosymbionts as they contribute to wood digestion by phagocytising wood particles. Possibly such invertebrates could also serve as vectors for *H. meleagridis*. In recent years *Reticulitermes* termite species gradually spread in France, especially the south and the west, including the province of Pays de Loire, with a large turkey population and numerous outbreaks of histomonosis during the hottest months (Callait-Cardinal et al., 2007). A speculative theory, proposed here for the first time, is that the litter (wood shavings) used in the farms may be contaminated with *H. meleagridis* infected termites.

A systematic study into the invertebrates that are associated with turkeys using PCR (and perhaps culture) could identify “new” intermediate hosts.

The existence of a cyst stage in combination with a mechanical vector, could be another explanation of flock to flock transmission of histomonosis in absence of *H. gallinarum*. During the experiments aiming at the optimization of the culture (Chapter 2), viable histomonads were lost when cultures were cooled to room temperature and returned to the incubator at 40°C after a few hours (unpublished data). Instead, histomonad-like forms were seen which were possibly previously also observed by others (Lund et al., 1967) They seemed to have a more rigid cell membrane, had more pronounced cellular organelles and sometimes possessed (the remainder of) a flagellum. These forms remained visible in culture for more than half a year, with no increase or decrease in cell counts. It was not possible to subculture these forms using Dwyer’s medium and standard conditions. It would be interesting to isolate these stages and to examine them by PCR. Another possibility

would be to inoculate turkeys orally and/or cloacally in order to assess their infectivity and examine whether it possibly concerns cyst stages of *H. meleagridis*. In a recent study, also a possible cyst stage was found by light microscopy in feces of a young infected chicken (Mielewczik et al., 2008). It was not definitely identified as *H. meleagridis* either, because it could not be sufficiently purified for examination by electron microscopy or other techniques.

Suggestions for future research

1. Refinement of infection models. Animal models usually represent a simplification of reality. Therefore the feasibility of an infection model using seeders and contact susceptible birds should be explored as it will mimic more closely a natural infection compared to the present models in which all birds are inoculated. It would be useful to include frequent cloacal swabbing for PCR and serology (ELISA) to determine transmission parameters.

2. Prevention of horizontal transmission. The possibilities to limit horizontal transmission during an histomonas outbreak have remained unexplored to date. Maybe certain types of litter are unfavourable for survival of histomonads in fresh droppings before cloacal drinking takes place. Alternatively, certain compounds that could be used to treat litter may proof useful at stopping the lateral spread of histomonads or as a preventive strategy.

3. Seroprevalence of *H. meleagridis* in commercial poultry. A sero-epidemiological study in chickens revealed a high seroprevalence of *H. meleagridis* in Dutch layers (unpublished data). The specificity of these findings needs to be confirmed by detection of *H. meleagridis* by PCR, culture and microscopic examination of smears at the time that most flocks seroconvert.

4. Molecular epidemiology. A systematic, international study on the occurrence of different ITS1-genotypes could prove useful. It would for instance be interesting to know if the relative frequent outbreaks on French turkey breeders farms are caused by strains with high virulence, and whether these differ from strains found in meat turkeys. A systematic registration of the background of the isolates (turkey or chicken origin, herd size, percent mortality in flock) is a prerequisite to identify ITS1-subtypes with a possible high virulence. Confirmation of virulence should be performed in pathogenicity studies. Relatively high virulent strains would be useful in stringent antihistomonal compound studies and vaccine challenge experiments.

5. *H. meleagridis* genotype III and *P. wenrichi*. *H. meleagridis* type III was identified as a novel genus closely related to *D. fragilis* and *H. meleagridis* by

molecular phylogeny. It was classified as likely being *P. wenrichi*, however, morphological data linking the molecular data to early descriptions of *P. wenrichi* were not fully satisfactory. This was also due to the fact that the organism could not be satisfactorily cultured using standard media. Further, staining of cryostored suspensions of cecal content has not been successful (unpublished data). Pheasants which have been reported to harbour *P. wenrichi* could be used as a renewed source for this protozoan. Cecal smears could be used for morphological studies and cecal contents could be examined using PCR and C-profiling.

6. Paromomycin. The clear antihistomonal *in vivo* prophylactic effect of paromomycin (Chapter 3.3) is very promising. Whether paromomycin could also be applied as metaphylactic or curative treatment needs to be examined.

Current situation

Considerable progress has been made by the scientific community with respect to detection, typing and control of histomonosis since this thesis was started in October 2003. New laboratory tools, like PCR, ELISA (this thesis) and molecular typing (this thesis) were developed, enabling further research into this devastating turkey disease. Promising antihistomonal compounds and antibiotics like paromomycin (this thesis) have been identified along with other potential effective control strategies like vaccination. Nevertheless, substantial additional research is required to study the transmission of the disease, to further examine vaccination, and to identify curative antihistomonals. The advances suggest that there is a good chance that eventually histomonosis will be brought under control again. Then *H. meleagridis* and histomonosis will hopefully retain the attention of scientists so that progress is maintained and re-emergence of the disease is avoided.

References

Bleyen, N., De, G.K., De, G.J., Goddeeris, B.M., 2007, Specific detection of *Histomonas meleagridis* in turkeys by a PCR assay with an internal amplification control. Veterinary Parasitology 143, 206-213.

Burch, D., Young, S., Watson, E., 2007, Treatment of histomonosis in turkeys with tiamulin. Veterinary Record 161, 864.

Callait-Cardinal, M.P., Leroux, S., Venereau, E., Chauve, C.M., Le Pottier, G., Zenner, L., 2007, Incidence of histomonosis in turkeys in France since the bans of dimetridazole and nifursol. Veterinary Record 161, 581-585.

Chan, F.T., Guan, M.X., Mackenzie, A.M., Diaz-Mitoma, F., 1994, Susceptibility testing of *Dientamoeba fragilis* ATCC 30948 with iodoquinol, paromomycin, tetracycline, and metronidazole. Antimicrobial Agents and Chemotherapeutics 38, 1157-1160.

Chossat, L., 2002, L'histomonose en production AOC 'Dinde Fermière de Bresse'. Essai de prevention par phytothérapie. Dissertation University of Lyon, France.

Dobell, C., 1940, Researches on the intestinal protozoa of monkeys and man. X. The life history of *Dientamoeba fragilis*: observations, experiments, and speculations. Parasitology 32, 417-461.

Dwyer, D.M., 1970, An improved method for cultivating *Histomonas meleagridis*. Journal of Parasitology 56, 191-192.

Dwyer, D.M., 1972a, Analysis of antigenic relationships among *Trichomonas*, *Histomonas*, *Dientamoeba*, and *Entamoeba*. I. Quantitative fluorescent-antibody methods. Journal of Protozoology 19, 316-325.

Dwyer, D.M., 1972b, Analysis of antigenic relationships among *Trichomonas*, *Histomonas*, *Dientamoeba*, and *Entamoeba*. II. Gel-diffusion methods. Journal of Protozoology 19, 326-332.

Dwyer, D.M., 1974, Analysis of antigenic relationships among *Trichomonas*, *Histomonas*, *Dientamoeba* and *Entamoeba*. III. Immunoelectrophoresis techniques. Journal of Protozoology 21, 139-145.

Frank, J.F., 1953, A note on the experimental transmission of enterohepatitis of turkeys by arthropods. Canadian Journal of Comparative Medicine 17, 230-231.

Gardner, T.B., Hill, D.R., 2001, Treatment of giardiasis. Clinical Microbiology Reviews, 14, 114-128.

Gerbod, D., Edgcomb, V.P., Noel, C., Delgado-Viscogliosi, P., Viscogliosi, E., 2000, Phylogenetic position of parabasalid symbionts from the termite *Calotermes flavicollis* based on small subunit rRNA sequences. International Microbiology 3, 165-172.

Gerbod, D., Edgcomb, V.P., Noel, C., Zenner, L., Wintjens, R., Delgado-Viscogliosi, P., Holder, M.E., Sogin, M.L., Viscogliosi, E., 2001, Phylogenetic position of the trichomonad parasite of turkeys, *Histomonas meleagridis* (Smith) Tyzzer, inferred from small subunit rRNA sequence. Journal of Eukaryotic Microbiology 48, 498-504.

Grabensteiner, E., Arshad, N., Hess, M., 2007, Differences in the *in vitro* susceptibility of mono-eukaryotic cultures of *Histomonas meleagridis*, *Tetratrichomonas gallinarum* and *Blastocystis* sp. to natural organic compounds. Parasitology Research 101, 193-199.

Grabensteiner, E., Hess, M., 2006, PCR for the identification and differentiation of *Histomonas meleagridis*, *Tetratrichomonas gallinarum* and *Blastocystis* spp. Veterinary Parasitology 142, 223-230.

Grabensteiner, E., Liebhart, D., Weissenbock, H., Hess, M., 2006, Broad dissemination of *Histomonas meleagridis* determined by the detection of nucleic acid in different organs after experimental infection of turkeys and specified pathogen-free chickens using a mono-eukaryotic culture of the parasite. Parasitology International 55, 317-322.

Graybill, H.W., Smith, T., 1920, Production of fatal blackhead in turkeys by feeding embryonated eggs of *Heterakis papillosa*. Journal of Experimental Medicine 31, 647-655.

Hafez, H.M., Hauck, R., 2006, Efficacy of a herbal product against *Histomonas meleagridis* after experimental infection of turkey poults. Archives of Animal Nutrition 60, 436-442.

Hafez, H.M., Hauck, R., Luschow, D., McDougald, L., 2005, Comparison of the specificity and sensitivity of PCR, nested PCR, and real-time PCR for the diagnosis of histomoniasis. Avian Diseases 49, 366-370.

Hauck, R., Hafez, H.M., 2007, Effect of coated plant extracts on *Histomonas meleagridis* and growth of bacteria *in vitro*. Avian Diseases 51, 880-883.

Hafez, H.M., Lofti, A., Comte, S., 2008, Efficacy of drinking water applications of paromomycin at different doses against Histomonas meleagridis in experimentally challenged turkey poults, 7th International Symposium on Turkey Diseases, Berlin, Germany, pp. 18-19.

Hegngi, F.N., Doerr, J., Cummings, T.S., Schwartz, R.D., Saunders, G., Zajac, A., Larsen, C.T., Pierson, F.W., 1999, The effectiveness of benzimidazole derivatives for the treatment and prevention of histomonosis (blackhead) in turkeys. Veterinary Parasitology 81, 29-37.

Hess, M., Kolbe, T., Grabensteiner, E., Prosl, H., 2006, Clonal cultures of *Histomonas meleagridis*, *Tetratrichomonas gallinarum* and a *Blastocystis* sp. established through micromanipulation. Parasitology 133, 547-554.

Hess, M., Liebhart, D., Grabensteiner, E., Singh, A., 2008, Cloned *Histomonas meleagridis* passaged *in vitro* resulted in reduced pathogenicity and is capable of protecting turkeys from histomonosis. Vaccine 26, 4187-4193.

Honigberg, B.M., Bennett, C.J., 1971, Lightmicroscopic observations on structure and division of *Histomonas meleagridis* (Smith). Journal of Protozoology 18, 687-697.

Hu, J., McDougald, L.R., 2004, The efficacy of some drugs with known antiprotozoal activity against *Histomonas meleagridis* in chickens. Veterinary Parasitology 121, 233-238.
Hu, J.H., McDougald, L.R., 2003, Direct lateral transmission of *Histomonas meleagridis* in turkeys. Avian Diseases 47, 489-492.
Huber, K., Chauve, C., Zenner, L., 2005, Detection of *Histomonas meleagridis* in turkeys cecal droppings by PCR amplification of the small subunit ribosomal DNA sequence. Veterinary Parasitology 131, 311-316.
Huber, K., Gouilloud, L., Zenner, L., 2007, A preliminary study of natural and experimental infection of the lesser mealworm *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) with *Histomonas meleagridis* (Protozoa: Sarcomastigophora). Avian Pathology 36, 279-282.
Johnson, E.H., Windsor, J.J., Clark, C.G., 2004, Emerging from obscurity: biological, clinical, and diagnostic aspects of *Dientamoeba fragilis*. Clinical Microbiology Reviews 17, 553-570.
Kotra, L.P., Haddad, J., Mobashery, S., 2000, Aminoglycosides : perspectives on mechanisms of action and strategies to counter resistance. Antimicrobial Agents and Chemotherapy, 44, 3249-3256.
Leveque, G., Cariou, N., Gallard, V., 2007. Outbreaks of histomoniasis in turkey breeders in France in recent years. International Symposium on Protozoal Infections in Poultry (Vienna, Austria), p. 8.
Liebhart, D., Grabensteiner, E., Hess, M., 2008, A virulent mono-eukaryotic culture of Histomonas meleagridis is capable of inducing fatal histomonosis in different aged turkeys of both sexes, regardless of the infective dose. Avian Diseases 52, 168-172.
Liebhart, D., Weissenbock, H., Hess, M., 2006, In-situ hybridization for the detection and identification of *Histomonas meleagridis* in tissues. Journal of Comparative Pathology 135, 237-242.
Lindquist, W.D., 1962, Some effects of paromomycin sulfate on blackhead in turkeys. American Journal of Veterinary Research 23, 1053-1056.
Lund, E.E., 1955, The progress of histomoniasis (blackhead) in turkeys as related to the size of the infective dose. Poultry Science 34, 127-130.
Lund, E.E., 1959, Immunizing action of a nonpathogenic strain of *Histomonas* against blackhead in turkeys. Journal of Protozoology 6, 182-185.
Lund, E.E., Augustin.Pc, Chute, A.M., 1967, *Histomonas meleagridis* after one thousand *in vitro* passages. Journal of Protozoology 14, 349-351.
Marouf, M., Lawrence, F., Croft, S.L., Robert-Gero, M., 1995. Ribosomes of *Leishmania* are a target for the aminoglycosides. Parasitology Research, 81, 421-425.
McDougald, L.R., 2005, Blackhead disease (histomoniasis) in poultry: a critical review. Avian Diseases 49, 462-476.
Mielewczik, M., Mehlhorn, H., Al-Quraishy, S., Grabensteiner, E., Hess, M., 2008, Transmission electron microscopic studies of stages of *Histomonas meleagridis* from clonal cultures. Parasitology Research 103, 745-750.
Reuvekamp, B., Mul, M., Fiks-Van Niekerk, T., 2008, Literature study on worms in laying hens. Report Animal Science Group No. 96 (Lelystad, the Netherlands), p. 24.
Schlessinger, D., 1988, Failure of aminoglycoside antibiotics to kill anaerobic, low-pH, and resistant cultures. Clinical Microbiology Reviews 1, 54-59.
Schroeder, R., Waldsich, C., Wank, H., 2000, Modulation of RNA function by aminoglycoside antibiotics. European Molecular Biology Organization Journal 19, 1-9.
Shaw, K.J., Rather, P.N., Hare, R.S., Miller, G.H., 1993, Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiological Reviews 57, 138-163.
Singh, A., Weissenbock, H., Hess, M., 2008, *Histomonas meleagridis*: immunohistochemical localization of parasitic cells in formalin-fixed, paraffin-embedded tissue sections of experimentally infected turkeys demonstrates the wide spread of the parasite in its host. Experimental Parasitology 118, 505-513.
Stark, D., Beebe, N., Marriott, D., Ellis, J., Harkness, J., 2006, Evaluation of three diagnostic methods, including real-time PCR, for detection of *Dientamoeba fragilis* in stool specimens. Journal of Clinical Microbiology 44, 232-235.
Tyzzer, E.E., 1920, Observations on the transmission of blackhead in turkeys - the common fowl as a source of infection. Journal of Medical Research 4, 219-237.

Tyzzer, E.E., 1934, Studies of histomoniasis, or "blackhead" infection, in the chickens and the turkey. Proceedings of the American Academy of Arts and Sciences 69, 189-264.
Tyzzer, E.E., 1936, A study of immunity produced by infection with attenuated culture-strains of *Histomonas meleagridis*. Journal of Comparative Pathology and Therapeutics 44, 285-303.
Vandenberg, O., Souayah, H., Mouchet, F., Dediste, A., van Gool, T., 2007, Treatment of *Dientamoeba fragilis* infection with paromomycin. Pediatric Infectious Disease Journal 26, 88-90.

Samenvatting

De parasiet *Histomonas meleagridis* kan een ernstige ziekte veroorzaken bij meestal hoenderachtige vogels zoals kalkoenen en kippen. Op kalkoenbedrijven kan de sterfte oplopen tot 100%! De ziekte is bekend onder de populaire naam “blackhead” (zwartekoppenziekte) of de wetenschappelijke naam “histomonosis” (voorheen “histomoniasis”). De parasiet is een eencellige microbe (protozo) die een blindedarmontsteking kan veroorzaken. Parasieten die vervolgens via de bloedbaan de lever bereiken, kunnen deze ernstig aantasten. Histomonas is erg kwetsbaar en kan nauwelijks buiten het lichaam van een vogel overleven als hij met de faeces wordt uitgescheiden. Histomonas kan echter de eieren van een andere veel voorkomende parasiet, de kleine spoelworm (*Heterakis gallinarum*) binnendringen. Deze eieren beschermen Histomonas tegen de ongunstige omstandigheden buiten de vogel en blijven jarenlang infectieus. Ook kan de regenworm nog een rol spelen in de verspreiding van de ziekte door het opnemen van de spoelwormeieren. Als de besmette regenwormen worden gegeten door gevoelige vogels infecteren deze dieren zich zowel met de kleine spoelworm als met Histomonas.

Sinds halverwege de jaren zeventig van de vorige eeuw kwam de ziekte nog nauwelijks voor bij commercieel pluimvee vanwege de beschikbaarheid van medicijnen waarmee de ziekte voorkomen of behandeld kon worden. Vanwege toegenomen bezorgdheid van overheden en burgers over de aanwezigheid van medicijnresiduen in voedsel van dierlijke oorsprong, en het feit dat het hier om mogelijk giftige of zelfs mogelijk kankerverwekkende stoffen ging, werden begin deze eeuw in Europa alle beschikbare medicijnen tegen histomonosis verboden. Omdat na het grotendeels verdwijnen van de ziekte er niet meer geïnvesteerd was in onderzoek, waren er nog geen alternatieve medicijnen voorhanden. Het was dan ook niet verbazingwekkend dat histomonosis opnieuw de kop opstak in diverse Europese landen. In Nederland deed de eerste nieuwe uitbraak zich voor in oktober 2003, en markeert ook het begin van het onderzoek dat tot dit proefschrift heeft geleid.

Hoofdstuk 1 bevat een algemene inleiding. Deze begint met een historisch overzicht omdat de geschiedenis juist bij deze aandoening zo’n belangrijke rol speelt. Al bij de eerste grote uitbraak in 1893 is er uitstekend onderzoek verricht en werd bijvoorbeeld de veroorzaker van de ziekte geïdentificeerd. De belangrijke rol van de kleine spoelworm werd in 1920 ontdekt. Nadat zich begin van deze eeuw nieuwe uitbraken voordeden, kwamen er ook fondsen beschikbaar voor het doen van wetenschappelijk onderzoek. Dankzij het feit dat er in de voorgaande dertig jaar nogal wat nieuwe moderne laboratoriumtechnieken beschikbaar waren gekomen, werd door verschillende onderzoekers op diverse terreinen snel vooruitgang geboekt. In dit hoofdstuk wordt verder de parasiet in detail beschreven, bij welke vogels deze voorkomt, hoe de levenscyclus in elkaar zit, hoe de ziekte zich manifesteert, welke laboratoriumtesten beschikbaar waren en kwamen, en welke middelen er beschikbaar waren.

Hoofdstuk 2 beschrijft hoe de kweekmethode voor de parasiet werd verbeterd. Dit was onder meer belangrijk om nieuwe medicijnen tegen histomonosis in het laboratorium te kunnen testen en om nieuwe laboratoriumtesten te kunnen ontwikkelen. De gevonden verbetering zat 'm vooral in de opbrengst aan parasieten, die met bijna een factor tien kon worden verhoogd. Verder kon de samenstelling van het medium worden vereenvoudigd, hetgeen tot een betere standaardisatie leidde.

In **hoofdstuk 3** werden daadwerkelijk nieuwe middelen tegen histomonosis getest, zowel in het laboratorium met behulp van de verbeterde kweekmethode, als in dierproeven. Omdat bij de start van dit onderzoek geen enkel bekend effectief middel meer toegelaten was voor gebruik in de pluimveehouderij, werden "alternatieve" producten gepropageerd als voertoevoegingen. Deze middelen bestonden vaak uit plantaardige extracten zoals knoflook en kaneel die weliswaar als antibacterieel bekend stonden, maar waarvan de werking tegen histomonosis onvoldoende bewezen was. Het bleek dat deze producten soms in de kweekmethode wel de parasiet in de groei remden, maar dat in de dierproeven geen enkele beschermende werking werd gezien. Wel bleek een antibioticum, paromomycine, zowel in de kweekmethode als in een dierproef kalkoenen goed te beschermen tegen de parasiet. Dat was een veelbelovend resultaat, maar voordat het gebruikt mag worden moet het nog als diergeneesmiddel in Nederland geregistreerd worden. Verder zou nog moeten worden onderzocht of het middel ook tijdens een uitbraak kan worden ingezet (behandeling van de ziekte in plaats van preventieve bescherming).

In **hoofdstuk 4** wordt de ontwikkeling van een nieuwe bloedtest (ELISA) beschreven waarmee afweerstoffen tegen *H. meleagridis* kunnen worden aangetoond. De toepassing van deze test zit 'm vooral in wetenschappelijk onderzoek naar de parasiet. Zo zijn kippen ook wel gevoelig voor de ziekte, maar leidt dit meestal tot veel minder uitval dan op kalkoenbedrijven. Het is bekend dat kippen een reservoir voor de parasiet vormen, maar het is niet goed bekend in welke mate de parasiet voorkomt bij kippen. Met de ontwikkelde bloedtest kan dit gemakkelijk en goedkoop worden onderzocht. De eerste resultaten laten zien dat de parasiet misschien wel wijder verbreid is dan gedacht. Een ander toepassingsgebied van de bloedtest is bij dierproeven waarbij vooral de verspreiding van de parasiet tussen dieren onderzocht wordt, en medicijnen of middelen die dat kunnen voorkomen.

Hoofdstuk 5 laat zien hoe een nieuwe DNA-test kan worden gebruikt om verschillende subtypes van *H. meleagridis* te onderscheiden. De bij mensen voorkomende parasiet *Dientamoeba fragilis* is nauw verwant aan *H. meleagridis*. De door het Amsterdams Medisch Centrum ontwikkelde methode voor DNA-typering zou mogelijk het onderscheid tussen kwaadaardige en onschuldige subtypes van *D. fragilis* kunnen maken. Bij een inventarisatie met die methode werden drie subtypes *H. meleagridis* gevonden. Eén type (III) was nogal afwijkend

van de andere twee en de kans bestaat dat dit in werkelijkheid de onschuldige protozo *Parahistomonas wenrichi* was. Subtypering zou vooral interessant zijn als zou blijken dat bepaalde subtypes veel kwaadaardiger zouden zijn dan andere.

Sinds de start van dit promotieonderzoek in oktober 2003, tijdens de eerste uitbraak van histomonosis in Nederland sinds jaren, is er veel vooruitgang geboekt door verschillende onderzoekers. Ondanks dat er nog veel onderzoek moet gebeuren is het de verwachting dat de histomonosis uiteindelijk weer onder controle komt. Het is te hopen dat onderzoekers zich ook dan zullen blijven bezighouden met deze parasiet zodat de ziekte niet over enige tijd opnieuw de kop opsteekt.

Dankwoord

Al veel promovendi voor mij hebben gewezen op het feit dat het dankwoord het meest gelezen onderdeel van een proefschrift is. De meeste proefschriftbladeraars nemen namelijk daar wat meer tijd voor, in de hoop hun naam erin aan te treffen. Evenals bij het samenstellen van de lijst van genodigden voor het promotiefeest is er een levensgroot risico dat de promovendus iemands bijdrage anders waardeert dan de persoon zelf, of, nog erger, iemand vergeet. In de wandelgangen heb ik gemerkt dat zoiets een jonge doctor nog járen wordt nagedragen. Daarom was ik zelfs al bij het begin van mijn promotieonderzoek daarop bedacht en heb zelfs aantekeningen bijgehouden. Mocht het desondanks zijn misgegaan dan hoop ik oprecht dat u het mij zal te laten weten; ik zal dan trachten het op gepaste wijze goed te maken (ik ben razend benieuwd wie dit durft).

Dit promotieonderzoek was niet mogelijk geweest als er geen financiering voor de onderzoeksprojecten zou zijn geweest. Het Productschap voor Vlees en Eieren (PVE) zag in dat histomonosis een tijdbom was (is!) voor de kalkoensector en heeft sinds 2003 jaarlijks een budget ter beschikking gesteld voor onderzoek. Ook de financiële bijdrage van de meeste kalkoenvoeder-producenten aan het onderzoek in hoofdstuk 2 - in feite de basis voor deze promotie - was essentieel. Daarnaast vond ik de periodieke besprekingen met de klankbordgroep om hen te informeren over de voortgang van het onderzoek en om te horen wat er speelt in de sector, altijd bijzonder informatief, kritisch, inspirerend en zinvol. Hopelijk was dat wederzijds. Jan Workamp nam voor die besprekingen telkens het initiatief.

Koen van Gussem (Huvepharma) bedank ik zeer dat ik hoofdstuk 3.3 in dit proefschrift mocht opnemen. Het lijkt me erg goed nieuws voor de kalkoensector dat er een middel met een bewezen effectiviteit tegen histomonosis beschikbaar lijkt te gaan komen. En ook biedt het aanknopingspunten in de zoektocht naar meer werkzame antibiotica.

Al in mijn Boxtelse tijd bij GD (1982-1995) was er regelmatig sprake van dat ik zou kunnen/mogen/moeten promoveren. Vooral Professor Martin Tielen moedigde mij daar bij herhaling toe aan. Helaas diende zich geen geschikt “groot” onderwerp aan, en zelf kon ik geen rode draad vinden in de vele onderwerpen waaraan ik werkte. Maar in die tijd heb ik wel veel op onderzoeksgebied geleerd o.m. dankzij het klimaatstal-project, waarbij ik als labtechneut bij enkele promotieonderzoeken intensief betrokken was. Ad van Exsel ben ik ondermeer dankbaar dat hij toen als hoofd laboratorium inzag dat een onderzoeksafdeling binnen het routinelab zinvol was, en dat ik die mocht gaan runnen. Die goeie ouwe tijd!

De belangrijkste persoon bij mijn promotie is co-promotor Wil Landman. Iedereen die hem kent, weet van zijn enorme passie voor de wetenschap. Bij GD moet hij degene zijn met vèruit de meeste wetenschappelijke publicaties. Tijdens mijn promotieperiode stuurde hij me zeker in het begin (droog je toch wat op, Wil?) vrijwel maandelijks een nieuw wetenschappelijk artikel van eigen hand. In de

frequente voortgangsbesprekingen die ik met hem had, zag hij bijna in elk idee(tje), resultaat(je) of discussie(tje) wel een mogelijkheid voor een artikel en/of vervolgonderzoek, maar om dit boekje binnen proporties te houden heb ik niet alles opgeschreven. Wil wordt op de GD-werkvloer soms ook wel gezien als een lastpak die “ons” met zijn ge-onderzoek heel veel extra werk bezorgt. Dat klopt overigens. En eerlijk gezegd dacht ik dat als eerste toen hij mij begin oktober 2003 e-mailde dat het hoog tijd werd dat GD wat aandacht zou gaan besteden aan “blackhead” i.v.m. het verbod op de laatste werkzame chemotherapeutica waarover de sector beschikte. Hij had wat gelden geregeld voor onderzoek en we moesten persé afreizen naar Athens, Georgia, VS, waar Professor Larry McDougald zowat als enige ter wereld nog serieus onderzoek deed aan *Histomonas meleagridis*. Maar die reis bleek het begin van iets moois (…). In dat naar GD-maatstaven lekker rommelige lab leerde ik de fijne kneepjes van de kweekmethode. Bepaald tot mijn ongenoegen zou een bepaald rijstpoeder uit de lokale Athense eco-shop, vooral omdat het vrij zou zijn van bestrijdingsmiddelen, een essentieel bestanddeel van het kweekmedium vormen. Ik gruwel van dergelijke vage claims zonder enig wetenschappelijk bewijs. Opmerkelijk genoeg was wetenschapper Wil desondanks graag bereid een kilo van dat witte poeder in zijn koffer mee naar Schiphol te smokkelen, toen ik hem vertelde daar tegenop te zien i.v.m. eerdere douaneperikelen. Met die kilo kunnen we de komende 10 jaar qua kweekwerk vooruit, zelfs als ik mijn eigen aanbeveling (hoofdstuk 2) opvolg om aanzienlijk meer (eco?)-rijstpoeder aan het kweekmedium toe te voegen. Mijn echtgenote is overigens van mening dat zelfs ecologisch rijstpoeder niet onbeperkt houdbaar zal blijken (rijstkoekjes mag ik er allang niet meer mee bakken). Voorlopig verzwelgen mijn histomonaadjes echter met gulzige gretigheid de “organic” rijstpartikels als ik ze daartoe aanzet. Een open eindje aan dit proefschrift is wat mij betreft nog wel om aan te tonen of (dat!) conventioneel rijstpoeder net zo goed is. Later, toen ik enige “alternatieve” middelen testte op anti-histomonas-activiteit (hoofdstuk 3), betichtte ’n enkele GD-collega mij zonder blikken of blozen van het doen van “zweverig” onderzoek (mij stijgt het bloed me opnieuw naar het hoofd). Ten onrechte uiteraard, immers voor de meeste producten was er wel degelijk een “theorie” over het werkingsmechanisme en zelfs als dat niet het geval zou zijn dan kan het onderzoek methodisch nog wel goed zijn. Maar ik dwaal af. Ik ben Wil erg dankbaar voor zijn scherpzinnigheid, zijn niet-aflatende enthousiasme en dat hij mijn lichtend voorbeeld als wetenschapper bleef binnen een steeds commerciëlere maatschappij. “Clowns to the left of me, jokers to the right. Here I am, stuck in the middle with you” (Gerry Rafferty, 1973). Bedankt Wil!

Mijn promotor, Professor Arjan Stegeman, ben ik uiteraard ook zeer erkentelijk. Arjan leerde ik kennen toen hij als veelbelovend onderzoeker bij de Gezondheidsdienst voor Dieren in Boxtel zijn promotieonderzoek startte. Dat moet wel een van de meest succesvolle onderzoeksprojecten in de veterinaire wereld ooit zijn geweest! Dankzij Arjan daalde tijdens zijn onderzoek de prevalentie van de

Ziekte van Aujeszky in het meest varkensdichte gebied van Europa van zo'n 50% naar minder dan 1%. Al voortbordurend op zijn aanbevelingen is de ziekte in Nederland inmiddels uitgeroeid. Heel erg bedankt, Arjan, dat je mijn promotor wilde zijn en ook dat je ons op het rechte onderzoekspad hield. Moeten we nog eens kijken naar een ideetje dat ik ooit tijdens jouw promotieonderzoek opperde? (ROC-analyses op basis van herd sensitivies/specificities i.p.v. testkarakteristieken voor individuen?).

I am very grateful to Professor Larry McDougald for allowing me to spend a week in his lab in Athens, Georgia in October 2003 to acquire sufficient "Fingerspitzengefühl" in histomonas culturing. My intention was to thoroughly prepare my own lab for a possible outbreak of histomonosis in the Netherlands, and it was rather surprising to me that this already happened while I was on my flight back! Apart from the technical part I also really enjoyed browsing and reading through all those sometimes ancient and-otherwise-hard-to-get papers of e.g. your histomonas expert predecessors Tyzzer and Lund that you have in your magnificent archive. I was also very pleased seeing your excellent review on *H. meleagridis* in print in 2005. During our visit Wil Landman and I encouraged you to write such a review, and perhaps this has contributed in some way to your decision to make the effort.

Jaap Wagenaar en Deborah van Doorn van de vakgroep Infectiologie, Faculteit Diergeneeskunde, Universiteit Utrecht wil ik hier ook noemen. Eigenlijk ter aanmoediging omdat hun vakgroep het laatste bastion lijkt te gaan worden van de veterinaire parasitologie in Nederland. Met Deborah reisde ik naar een protozoa-symposium in Wenen. Tijdens het inauguratiefeest van Jaap bleek dat vrijwel alle Nederlandse veterinaire parasitologen in meer of mindere mate de Russische taal beheersen (en ook ik ben al weer aan mijn 5^{e} jaar in de avonduren bezig om wat Russisch te leren). Dus ik neem aan dat Deborah en Jaap ook aan het studeren zijn?

Dan wil ik nog de nodige GD-ers bedanken die bijdroegen aan mijn promotie-onderzoek. Allereerst mijn baas Kees van Maanen, die me genoeg ruimte liet tussen andere klussen door, mijn baas' baas Frens Westenbrink die mij bij elk denkbare gelegenheid enorm aanmoedigde en GD-directeur Anton Pijpers die bij het begin zijn fiat gaf en mijn periodieke updates altijd op prijs stelde. Maar ik dank natuurlijk vooral diegenen die letterlijk in actie kwamen, zoals sectiezaal-medewerkers Gert-Jan en Dave, dierverzorgers Johan, Ad en Machiel, hybridoma-kweekster Annelies, immunohistochemiër José, histoloog Johan Wensink en statisticus Wim. En niet te vergeten buur(t)vrouw en collega Gaby (en in casu al haar collega-virologen) die iedere keer weer bereid was mij eventueel te gaan redden als ik weer eens histomonas-isolaatjes in/uit de stikstofopslag moest krijgen. Zeer onlangs heb ik het zelf gebracht tot gediplomeerd stikstofslachtoffer-redder. Tijdens het examen bleek het beschikbare zuurstofmasker maar nèt te passen….

Mijn directe collega's van R&D ga ik hier maar niet uitvoerig bedanken, want die zeurden echt jarenlang over die ietwat onwelriekende histomonas-kweken. In dit verband heb ik Kees overigens eens bijna moeten reanimeren toen hij zonder veel omhaal een flinke neusteug nam uit een kweekflesje n.a.v. de aanhoudende R&D-klachten. Tja. Wel kon men enige waardering opbrengen voor de "alternatieve" middelen (kaneel, knoflook) die ik gedurende een bepaalde tijd aan de kweekjes toevoegde (er stegen heerlijke luchten op uit het lab-kastje waarin ik die materialen bewaarde). In het laatste jaar heeft Constance toch nog het nodige ELISA-werk voor mij gedaan (waarvan de resultaten uiteindelijk toch niet in dit proefschrift staan). Nou vooruit: de rest van R&D (Huub, Ingrid, Jan, Jolanda, Kees, Rianne, Roy en Thea) dank ik, vol goede moed, alvast voor het "stukje" tijdens het promotiefeest! (R&D's optreden vreest een promovendus nog meer dan de verdediging).

Naast Wil en Jan en droegen andere PGZers ook bij: Herman, Hilde, Naomi, Rob, Wijnand. En Anneke moedigde me nogal eens aan. Ik dank ook mijn metgezellen van "epi-unit", hèt "jonge-onderzoekers"-clubje binnen GD. Daar moeten onderzoekers uit alle GD-geledingen, mits lid, met de billen bloot en worden hun presentaties c.q. onderzoeksideeën vooral op epidemiologisch en statistisch gebied aan kritische vragen onderworpen. Een fijne leerschool. Epiërs van het eerste uur zijn Ad, Anneke, Chris, Henriëtte, Lammert, Otlis, en Peter (bij wiens eigen promotie ik ooit als paranimf mocht "oefenen"). Statisticus Wim zit nu de club voor en heeft verder mij in mijn onderzoek enkele malen op zijn vakgebied de juiste afslag laten nemen.

De wetenschappelijke stafleden van het lab toonden zich fijne collega's die met enige regelmaat wel eens wilden weten of het deze analist nou echt zou gaan lukken en of het al een beetje opschoot. Nou, Anja, Eric, Gerard, Guillaume, Jan, Jet en Sjaak: het boekje is af. In dit verband is het ook jammer dat GD alleen een parasitoloog op afstand heeft. Fred Borgsteede, werkzaam bij ASG, is ook part-time parasitoloog bij GD. Hij suggereerde me in de wandelgangen ook maar eens een artikel naar Veterinary Parasitology te sturen in plaats van telkens dat weliswaar hoog-gerate Avian Pathology. Nou Fred, twee dus! Indertijd reageerde je trouwens bliksemsnel op de eerste recente uitbraak van histomonosis in Nederland. Via een ingezonden brief in *De Volkskrant* van 31 oktober 2003, nuanceerde je het alarmerende bericht van een week eerder dat de "zwartekoppenziekte" het voortbestaan van de gehele kalkoensector zou bedreigen. Dat was in zekere zin terecht, maar zie ook mijn discussie!

Als laatste GDer wil ik Thijs Roumen bedanken die mij jaren geleden een oude (jaren 30) histomonosis-lever op "sterk water" cadeau deed. Tot nu toe heb ik de verleiding kunnen weerstaan om wat materiaal uit een laesie te prikken, om te proberen of ik er DNA uit kan isoleren teneinde de stam via C-profilering te genotyperen. Maar wie weet!

Mijn broer Hans en vriend René namen zonder aarzelen de uitnodiging aan om mij als paranimf bij de verdediging te ondersteunen. Moreel dan, want sinds kort is dat in Utrecht een uitsluitend ceremoniële functie. Toen ikzelf ooit als paranimf aantrad, was ik zenuwachtiger dan ooit. Want stel bijvoorbeeld dat die promovendus van de spanning in huilen uitbarst (zoals ik ooit als afstudeerbegeleider van een HLO-student meemaakte)!

Mijn schoonzus Nanja ontwierp de prachtige omslag van dit proefschrift. Zij is grafisch ontwerpster (kunstacademie) en won al eens de prijs bij de jaarlijkse verkiezing van Het Mooiste Boekenomslag (en werd nog vaker genomineerd). Eerder al tekende zij, letterlijk en figuurlijk, voor het omslag van mijn schaakboekje *Pawn promotion*. Het is geweldig hoe je dat doet, Nanja. Ik weet ongeveer wat ik leuk zou vinden, maar mijn eigen prutserige aanzet ziet er, zelfs in mijn ogen, niet uit. Met enkele kordate strepen en/of verschuivingen maak je het dan precies goed, maar besteed je nog menig uurtje aan de details. Vakvrouwschap is meesterschap!

Dorette is de belangrijkste persoon in mijn leven. Zij leidt met zachte hand een post-modern huishouden van Jan Steen, waarbij onze zonen Rens en Tim zo ongeveer vergroeid zijn met hun computer(passie) en ik ook wel eens wat tijd doorbreng achter de PC. Vooral de ietwat uit de hand gelopen schaak-eindspelstudiehobby slokte vrijwel al mijn vrije tijd gedurende de afgelopen 20 jaar op. Lieve Dorette, zonder jou was dit alles nooit gelukt. Eigenlijk zouden we onze GD-banen op moeten zeggen, de kids op kamers sturen, de schaakbibliotheek verkopen en samen afreizen naar een buitenlands lab waar het jouw beurt is om te promoveren. Doen?

Helaas kan mijn vader de afronding van mijn promotie niet meer meemaken. Hij zou best wel trots op mij zijn geweest. Vlak voordat hij een jaar geleden overleed wenste hij me er nog veel succes mee toe en twijfelde er niet aan dat het me zou gaan lukken. Ik koester fijne herinneringen aan een congresbezoek in 2006. Omdat mijn moeder nooit mee durfde op vliegreizen, had ik mijn vader eens beloofd dat ik hem bij een eerstvolgend congresbezoek zou meenemen. Toen ik hem begin 2006 belde: “Ok Pa, het wordt het jaarlijkse Amerikaanse pluimvee-congres”, was hij enthousiast en bleef dat toen ik hem daarna vertelde dat het congres toevalligerwijs dat jaar (telkens een andere staat) in Hawaï plaats zou grijpen. De 25-urige reis doorstond hij glansrijk, en hij heeft toen veel meer van Honoloeloe en Waikiki Beach gezien dan ik (die voornamelijk in de congreszaal vertoefde).

Dit proefschrift draag ik op aan mijn moeder. Lieve Ma, ik doe dat niet alleen omdat het afgelopen jaar voor jou bijzonder triest en zwaar is geweest, en ook zijn wij allemaal bevreesd voor hetgeen nog in het verschiet ligt. Maar ik dank je vooral om jouw onvoorwaardelijke steun in alles wat ik toen nu toe gedaan heb. Die steun voel ik in mijn hart en zal me ook in de toekomst helpen om moeilijke momenten te doorstaan.

Curriculum vitae

Henricus Marinus Johannes Franciscus (Harold) van der Heijden werd op 18 december 1960 geboren te Veghel. Na in 1978 het HAVO-diploma behaald te hebben, rondde hij in 1981 een opleiding HBO-B biochemie aan de Hogere en Middelbare Laboratoriumschool in Oss met succes af. In het kader van deze opleiding werd een stage en een afstudeeropdracht (ontwikkeling van monoklonale antistoffen tegen het Hepatitis B virus) gedaan bij Organon te Oss. Na het vervullen van de militaire dienstplicht (meteorologische afdeling van de artillerie) trad hij op 1 december 1982 dienst bij de Gezondheidsdienst voor Dieren (GD) in Zuid-Nederland te Boxtel. In de eerste jaren introduceerde hij een door het Instituut voor Veeteeltkundig onderzoek (IVO) te Zeist ontwikkelde progesterontest bij alle GD-laboratoria. Dit betekende o.m. een part-time detachering bij het IVO gedurende 2 jaar. Daarna werd onder zijn leiding een R&D-laboratorium in Boxtel gestart waar hij verantwoordelijk was voor het ontwikkelen van diverse testen en betrokken was bij wetenschappelijk onderzoek in de klimaatstal voor varkens en andere promotieonderzoeken. Sinds de fusie van de GD's is hij sinds 1995 bij de R&D-afdeling in Deventer werkzaam als onderzoeksanalist. In oktober 2003 startte hij met het histomonas-werk maar pas in 2005 werd besloten om een promotietraject in te zetten.

List of publications

Journals (peer-reviewed)

Bart, A., Van der Heijden, H.M.J.F., Greve, S., Speijer, D., Landman, W.J.M., Van Gool, T., 2008, The intragenomic variation in the ITS-1 region of *Dientamoeba fragilis* as a molecular epidemiological marker. Journal of Clinical Microbiology 46, 3270-3275.

Feberwee, A., Hampson, D.J., Phillips, N.D., La, T., van der Heijden, H.M.J.F., Wellenberg, G.J., Dwars, R.M., Landman, W.J.M., 2008, Identification of *Brachyspira hyodysenteriae* and other pathogenic *Brachyspira* species in chickens from laying flocks with diarrhea or reduced production or both. Journal of Clinical Microbiology 46, 593-600.

Van der Heijden, H.M.J.F., Landman, W.J.M., 2008, *In vitro* effect of herbal products against *Histomonas meleagridis*. Veterinary Parasitology 154, 1-7.

Van der Heijden, H.M.J.F., Landman, W.J.M., 2008, *In vivo* effect of herbal products against *Histomonas meleagridis* in turkeys. Avian Pathology 37, 45-50.

Van der Heijden, H.M.J.F., Landman, W.J.M., 2007, Improved culture of *Histomonas meleagridis* in a modification of Dwyer medium. Avian Diseases 51, 986-988.

Van der Heijden, H.M.J.F., Landman, W.J.M., Greve, S., Peek, R., 2006, Genotyping of *Histomonas meleagridis* isolates based on internal transcribed spacer-1 sequences. Avian Pathology 35, 330-334.

Van der Heijden, H.M.J.F., McDougald, L.R., Landman, W.J.M., 2005, High yield of parasites and prolonged in vitro culture of *Histomonas meleagridis*. Avian Pathology 34, 505-508.

Van der Heijden, H.M.J.F., Bakker, J., Elbers, A.R., Vos, J.H., Weyns, A., De Smet, K., McOrist, S., 2004, Prevalence of exposure and infection of *Lawsonia intracellularis* among slaughter-age pigs. Research in Veterinary Science 77, 197-202.

Van der Heijden, H.M.J.F., 2001, First international ring trial of ELISAs for *Salmonella*-antibody detection in swine. Berliner und Münchener Tierärztliche Wochenschrift 114, 389-392.

Van der Wolf, P.J., Elbers, A.R., Van der Heijden, H.M.J.F., Van Schie, F.W., Hunneman, W.A., Tielen, M.J., 2001, *Salmonella* seroprevalence at the population and herd level in pigs in the Netherlands. Veterinary Microbiology 80, 171-184.

Van der Wolf, P.J., Lo Fo Wong, D.M., Wolbers, W.B., Elbers, A.R., Van der Heijden, H.M.J.F., Van Schie, F.W., Hunneman, W.A., Willeberg, P., Tielen, M.J.M., 2001, A longitudinal study of *Salmonella enterica* infections in high- and low-seroprevalence finishing swine herds in the Netherlands. Veterinary Quarterly 23, 116-121.

Van der Wolf, P.J., Van Schie, F.W., Elbers, A.R., Engel, B., Van der Heijden, H.M.J.F., Hunneman, W.A., Tielen, M.J., 2001, Administration of acidified drinking water to finishing pigs in order to prevent *Salmonella* infections. Veterinary Quarterly 23, 121-125.

Van der Wolf, P.J., Wolbers, W.B., Elbers, A.R., Van der Heijden, H.M.J.F., Koppen, J.M., Hunneman, W.A., Van Schie, F.W., Tielen, M.J., 2001, Herd level husbandry factors associated with the serological *Salmonella* prevalence in finishing pig herds in the Netherlands. Veterinary Microbiology 78, 205-219.

Elbers, A.R.W., Rambags, P.G.M., Van der Heijden, H.M.J.F., Hunneman, W.A., 2000, Production performance and pruritic behaviour of pigs naturally infected by *Sarcoptes scabiei* var. suis in a contact transmission experiment. Veterinary Quarterly 22, 145-149.

Stegeman, J.A., Rambags, P.G.M., Van der Heijden, H.M.J.F., Elbers, A.R.W., Hunneman, W.A., 2000, Experimental quantification of the transmission of *Sarcoptes scabiei* var. suis among finishing pigs. Veterinary Parasitology 93, 57-67.

Van der Heijden, H.M.J.F., Rambags, P.G.M., Elbers, A.R.W., Van Maanen, C., Hunneman, W.A., 2000, Validation of ELISAs for the detection of antibodies to *Sarcoptes scabiei* in pigs. Veterinary Parasitology, 95-107.

Stegeman, J.A., De Jong, M.C., Van der Heijden, H.M.J.F., Elbers, A.R., Kimman, T.G., 1997, Evaluation of tests for detection of antibodies to Aujeszky's disease (pseudorabies) virus glycoprotein E in the target populations. Veterinary Microbiology 55, 107-111.

Stegeman, J.A., De Jong, M.C.M., Van der Heijden, H.M.J.F., Elbers, A.R.W., Kimman, T.G., 1996, Assessment of the quality of tests for the detection of antibodies to Aujeszky's disease virus glycoprotein gE in a target population by the use of receiver operating characteristic curves. Research in Veterinary Science 61, 263-267.

Barrow, P., Desmidt, M., Ducatelle, R., Guittet, M., Van der Heijden, H.M.J.F., Holt, P.S., Huis in 't Veld, J., McDonough, P.L., Nagaraja, K.V., Porter, R.E., Proux, K., Sisak, F., Staak, C., Steinbach, G., Thorns, C., Wray, C., Van Zijderveld, F., 1996, World Health Organisation - supervised interlaboratory comparison of ELISAs for the serological detection of *Salmonella enterica* serotype Enteritidis in chickens. Epidemiology and Infection 117, 69-77.

Coppens, M.L.G., Remmen, J.W.A., Van der Heijden, H.M.J.F., 1987, Which possibilities does the milk progesterone test in dairy cattle offer in the field? Tijdschrift voor Diergeneeskunde 112, 519-530.

Abstracts and posters

Feberwee, A., Hampson, D.J., Phillips, N.D., La, T., Van der Heijden, H.M.J.F., Wellenberg, G.J., Landman, W.J.M., 2008, Survey of *Brachyspira* spp. in Dutch poultry and the isolation of a *Brachyspira hyodysenteriae*-like spirochete. 15th World Veterinary Poultry Congress (Beijing, China), p. 260.

Mantini, C., Dalia-Cornette, J., Noda, S., Van der Heijden, H.M.J.F., Dei-Cas, E., Landman, W.J.M., Ohkuma, M., Viscogliosi, E., 2008, Molecular identification and phylogenetic relationships of trichimonad isolates of galliform birds inferred from nuclear small subunit rRNA gene sequences and identification of a novel genotype likely corresponding to *Parahistomonas wenrichi*. 7th International Symposium on Turkey Diseases, (Berlin, Germany), p. 23.

Van der Heijden, H.M.J.F., Landman, W.J.M., 2008, *In vitro* and *in vivo* activity of herbal products against *Histomonas meleagridis*. 15th World Veterinary Poultry Congress (Beijing, China), p. 268.

Feberwee, A.H., Hampson, D.J., Phillips, N.D., La, T., Van der Heijden, H.M.J.F., Wellenberg, G.J., Landman, W.J.M., 2007, Survey of *Brachyspira* spp. in Dutch poultry and the isolation of a *Brachyspira hyodysenteriae* like spirochaete. 4th International Conference on Colonic Spirochaetal Infections in Animals and Humans (Prague, Czech Republic), p. 36.

Maassen, C., Achterberg, R., Engel, B., Van der Giessen, J., Teunis, P., Van der Heijden, H.M.J.F., Van der Wolf, P.J., Haughey, S., Molina, V., Larrieu, E., Peralta, J.L., Döpfer, D., 2007, Evaluation of serological tests for *Trichinella* infections in pigs. 7th International Safepork Symposium (Verona, Italy). pp. 211-213.

Van der Heijden, H.M.J.F., Landman, W.J.M., 2007, *In vitro* and *in vivo* activity of herbal products against *Histomonas meleagridis*. International Symposium on Protozoal Infections in Poultry (Vienna, Austria), pp. 22-23.

Van der Heijden, H.M.J.F., Landman, W.J., Greve, S., Peek, R., 2006, C-profiling: a novel technique for genotyping *Histomonas meleagridis*. 6th International Symposium on Turkey Diseases (Berlin, Germany), pp. 205-209.

Van der Heijden, H.M.J.F., Landman, W.J.M., Greve, S., Peek, R., 2006, *Histomonas meleagridis*: genotyping of isolates using a novel technique: C-profiling. 50th Conference of American Association of Avian Pathologists (Honolulu, USA), p. 163.

Landman, W.J., Van der Heijden, H.M.J.F., McDougald, L.R., 2005, High yield of parasites and prolonged *in vitro* culture of *Histomonas meleagridis*. 54th Western Poultry Disease Conference (Vancouver, Canada), pp. 35-36.

Landman, W.J., McDougald, L.R., Van der Heijden, H.M.J.F., 2004, Experimental infestation of turkeys and chickens with a Dutch field isolate of *Histomonas meleagridis*. 5th International Symposium on Turkey Diseases (Berlin, Germany), pp.53-54.

De Wit, J.J., Mekkes, D.R., Van der Heijden, H.M.J.F., Heijmans, J.F., 2003, Sequencing of Dutch IBDV strains involved in clinical outbreaks in the winter of 2003. Cost Action 839: Immunosuppressive Viral Diseases (Perugia, Italy), pp. 28-32.

Van der Wolf, P.J., Wolbers, W.B., Elbers, A.R., Van der Heijden, H.M.J.F., Koppen, J.M., Hunneman, W.A., Van Schie, F.W., Tielen, M.J.M., 2001, Risk factors for *Salmonella* in finishing pigs in the Netherlands. First Global Virtual Conference on Swine Health, pp. 1-10.

Bakker, J., Van der Heijden, H.M.J.F., De Smet, K., Vos, J., 2000, Comparison of Laboratory techniques for the diagnosis of porcine proliferative enteropathy (PPE) in fattening pigs. International Pig Veterinary Society Congress (Melbourne, Australia), p. 25.

Mortimer, I., Van der Heijden, H.M.J.F., Henderson, L.E., Thomson, J.R., Lawrence, K., 2000, Proficiency testing among European laboratories using an immuno-fluorescent antibody test (IFAT) for the detection of antibodies against Lawsonia intracellularis in pigs. International Pig Veterinary Society Congress (Melbourne, Australia), p. 77.

Rambags, P.G.M., Elbers, A.R.W., Van der Heijden, H.M.J.F., Hunneman, W.A., 2000, The Dutch mange free (*Sarcoptes scabiei* var. suis) certification programme: evaluation and update after a transmission experiment. International Pig Veterinary Society Congress (Melbourne, Australia), p. 267.

Van der Heijden, H.M.J.F., 2000, Validation of veterinary immunoassays: evaluation of field samples. Advanced Methods for Test Validation and Interpretation in Veterinary Medicine (Berlin, Germany), pp. 96-97.

Van der Heijden, H.M.J.F., Mekkes, D., De Wit, J.J., 2000, Validation of veterinary immunoassays: risk analysis and study design. Advanced Methods for Test Validation and Interpretation in Veterinary Medicine (Berlin, Germany), p. 95.

Van der Wolf, P.J., Van Schie, F.W., Elbers, A.R.W., Engel, B., Van der Heijden, H.M.J.F., Hunneman, W.A., Tielen, M.J.M., 2000, Addition of organic acids to the drinking water of finishing pigs: preliminary results of an intervention study to prevent *Salmonella infections*. International Pig Veterinary Society Congress (Melbourne, Australia), p. 214.

Bol, C.J., Voets, M.T., Elbers, A.R.W., Stegeman, J.A., Van Nes, A., Van der Heijden, H.M.J.F., Hunneman, W.A., 1998, Evaluation of pseudorabies virus eradication program in the Netherlands: an inventory of possible setbacks. International Pig Veterinary Society Congress (Birmingham, United Kingdom), p. 181.

Duinhof, T.F., De Jong, M.F., Bakker, J., Van der Heijden, H.M.J.F., De Ridder, E., 1998, Diagnosis of proliferative enteropathy with polymerase chain reaction in faeces on Dutch pig farms. Results of a pilot study. International Pig Veterinary Society Congress (Birmingham, United Kingdom), p. 117.

Rambags, P.G.M., Vesseur, P.C., Van der Heijden, H.M.J.F., 1998, Mange (*Sarcoptes scabiei* var suis) eradication programme and possibilities for certification in Dutch pig farms. International Pig Veterinary Society Congress (Birmingham, United Kingdom), p. 123.

Van der Heijden, H.M.J.F., Boleij, P.H.M., Loeffen, W.L.A., Bongers, J.H., Van der Wolf, P.J., Tielen, M.J.M., 1998, Development and validation of an indirect ELISA for the detection of antibodies against Salmonellae in swine. International Pig Veterinary Society Congress (Birmingham, United Kingdom), p. 122.

Van der Heijden, H.M.J.F., Rambags, P.G.M., Vesseur, P.C., 1998, Development and validation of an ELISA for the detection of antibodies against *Sarcoptes scabiei* to be used in an eradication programme. International Pig Veterinary Society Congress (Birmingham, United Kingdom), p. 69.

Van der Wolf, P.J., Elbers, A.R.W., Wolbers, W.B., Koppen, J.M.C.C., Van der Heijden, H.M.J.F., Van Schie, F.W., Hunneman, W.A., Tielen, M.J.M., 1998, Risk Factors for Salmonella in slaughter pigs in The Netherlands. International Pig Veterinary Society Congress (Birmingham, United Kingdom), p. 68.

Vesseur, P.C., Rambags, P.G.M., Van der Heijden, H.M.J.F., 1998, *Sarcoptes scabie*i var. suis status and eradication on seven combined farrow to finish farms, the base for an eradication programme. International Pig Veterinary Society Congress (Birmingham, United Kingdom), p. 121.

Van der Wolf, P.J., Wolbers, W.B., Elbers, A.R.W., Van der Heijden, H.M.J.F., Van Schie, F.W., Hunneman, W.A., Tielen, M.J.M., 1997, Blood sampling at two slaughterhouses and serological screening of *Salmonella* infections in swine using an indirect ELISA. Second International Symposium on Epidemiology and Control of Salmonella in Pork (Copenhagen, Denmark), pp. 199-202.

Van der Heijden, H.M.J.F., 1995, The development and application of LPS-ELISAs to detect *Salmonella* infections in swine. Symposium on the Diagnosis of Salmonella Infections (Reno, USA), pp. 88-95.

Van der Heijden, H.M.J.F., 1994, Effect of periodate oxidation of LPS antigens on the specificity of *Salmonella* serogroups B and D ELISAs for serological screening of poultry. USAHA Salmonella Committee (Grand Rapids, USA), p. 19.

Stegeman, J.A., Loeffen, W.L.A., Van der Heijden, H.M.J.F., 1993, Determination of the Aujeszky's disease virus gI-serostatus of pigs by testing a single droplet of whole blood. International Pig Veterinary Society Congress (Bangkok, Thailand), p. 94.

Bongers, J., Driessen, Y., Moraal, S., Van der Heijden, H.M.J.F., 1992, Comparison of Rappaport- Vassiliadis medium and semisolid enrichment media for isolation of *Salmonella* from animal faeces and animal products. VIth International Symposium of the World Association of Veterinary Laboratory Diagnosticians (Lyon, France), p. 14.

Bongers, J., Gruythuizen, K., Driessen, Y., Van der Heijden, H.M.J.F., 1992, Proficiency testing in clinical bacteriology for animal health services as a tool in external quality control. VIth International Symposium of the World Association of Veterinary Laboratory Diagnosticians (Lyon, France), p. 32.

Van den Wijngaard, J.C., Van der Heijden, H.M.J.F. 1991, ELISA for serological screening of poultry for salmonellae, including *Salmonella enteritidis*. 10th Symposium on the Quality of Poultry Meat and 4th Symposium on the Quality of Eggs and Egg Products (Doorwerth, the Netherlands).

Van der Heijden, H.M.J.F., Van den Wijngaard, J.C., 1991, Salmonella- ELISA for serological screening of poultry. Symposium on the Diagnosis and Control of Salmonella (San Diego, USA), pp. 149-156.