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ScienceDirect
Parasitology International 55 (2006) 317–322
www.elsevier.com/locate/parint

Short communication

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

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Received 8 February 2006; received in revised form 16 June 2006; accepted 20 July 2006

Abstract

Histomonas meleagridis, a flagellated protozoan parasite, is the causative agent of histomonosis (syn. histomoniasis, blackhead) in turkeys and chickens. The organs primarily affected by the parasite are the caeca and the liver. Until now, only few reports exist in which the parasite has been diagnosed in tissues other than those mentioned above. Hence, the aim of this study was to perform a systematic investigation of various organs of turkeys and specified pathogen-free chickens following an experimental infection with a mono-eukaryotic culture of *Histomonas meleagridis* in order to determine the dissemination of the flagellate in infected birds. Molecular methods like PCR and in situ hybridization were used for this purpose. For the first time, the DNA of the parasite could be detected in 13 different organs of infected turkeys by PCR including the proventriculus, duodenum, jejunum, caeca, pancreas, bursa of Fabricius, liver, kidney, spleen, heart, lung, thymus and the brain. Most of these findings were further confirmed by in situ hybridization. In contrast to the turkeys that all died shortly after the infection, all of the chickens survived without displaying any clinical symptoms. Even at necropsy, only mild pathological changes were observed in the caeca. Nevertheless, the parasite could also be detected in various organs of these birds, namely the caeca, bursa of Fabricius, kidney, heart and the brain.

Keywords: *Histomonas meleagridis*; PCR; In situ hybridization; Dissemination; Turkeys; spf chickens

The flagellate *Histomonas meleagridis* causes histomonosis (syn. histomoniasis, blackhead disease), a parasitic disease of economic importance in turkeys and chickens [1]. Typical clinical symptoms like sulfur-coloured droppings, drowsiness, and weakness can often be observed in infected turkeys which are most susceptible for the disease [2]. In chickens, however, the predominant symptoms include a slight increase in mortality and a decreased laying performance [3,4]. The caeca and liver are the main target organs of *Histomonas meleagridis* in infected birds giving the disease one of its names (infectious enterohepatitis) [2]. Until now, there are only few reports regarding the detection of *Histomonas meleagridis* in organs other than caeca and liver in turkeys and chickens [3–12]. The diagnosis of the infection in these cases was mainly based on necropsy findings and confirmed by histopathological investigations using different staining techniques as well as cytology. Anyhow, diagnosis of the parasite in infected tissues by conventional histological staining techniques or cytology can be hampered as it is difficult to distinguish the histomonads from fungi or other protozoa [13]. Recently, the detection of histomonad DNA in faecal samples and various tissues of meat turkeys after natural and experimental infection with *Histomonas meleagridis* was reported by conventional and real-time PCR [14,15]. However, no study exists so far in which the dissemination of *Histomonas meleagridis* has been examined in detail in turkeys and chickens after experimental infection with a mono-eukaryotic isolate via the cloacal route. Therefore, the aim of this study was to detect the flagellate by molecular methods like

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1383-5769/$ - see front matter © 2006 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.parint.2006.07.004

PCR and in situ hybridization (ISH) in various selected organs of artificially infected turkeys and chickens investigating the same range of tissues. These methods allow a definite diagnosis of the presence of at least the nucleic acid of the parasite in organ samples.

For the infection experiment a mono-eukaryotic culture of *Histomonas meleagridis* was used. It was established out of the caecal contents and faecal material of a bronze turkey that died at the age of about 20 weeks due to histomonosis. The procedure was described recently in detail [16]. Briefly, approximately 1 g of caecal content was placed in 9 ml of Medium 199 + Earle's salts + L-Glutamine + 25 mM HEPES + L − Amino acids (Gibco™) containing 11 mg of rice starch (Sigma Aldrich), 10% foetal calf serum (FKS), antibiotics (200 IE penicillin and 200 μg streptomycin per ml medium) and an antimycoticum (2.5 μg amphotericin B/ml medium). Passages were performed every second day up to 7 passages when micromanipulation was performed. Single parasites were selected randomly with a capillary and parasites were released into an Eppendorf tube. After the isolation procedure the microbes were incubated at 40 °C up to 4 days in individual Eppendorf tubes. Any positive or suspicious colony was transferred into 9.7 ml of the standard medium described above in a 50 ml plastic tube (Sarstedt) and incubated as described above.

Tissue samples used in this study originated from two separate infection experiments in which two-week-old turkeys as well as specified pathogen-free (spf) chickens were infected via the cloaca with a cloned isolate of *Histomonas meleagridis* (*Histomonas meleagridis*/Turkey/Austria/2922-C6/04) for the first time [17]. Briefly, 18 two-week-old birds (either turkeys or spf chickens) were used in two different animal trials. The birds were separated into two groups: the control group consisting of four birds (no. 1–4) and the infection group consisting of 14 birds (no. 5–18) of which 10 birds were infected and 4 birds were kept as in-contact birds. The two groups were housed in two separate rooms after infection with 380,000 histomonads.

For PCR and ISH, tissue samples of the proventriculus, duodenum, jejunum, caeca, pancreas, bursa of Fabricius, liver, kidney, spleen, heart, lung, thymus and brain were obtained from the turkeys and chickens described above. According to the design of the experiments the organs originated either from infected or in-contact birds kept in the same room. In addition, organs from separately housed non-infected birds were included as control. These birds were free of any detectable flagellate as demonstrated by haematoxylin and periodic acid-Schiff staining of tissues. In order to avoid cross contamination during necropsy, disposable tweezers and autoclaved scissors were used during the whole sampling procedure.

DNA was extracted from tissue samples employing the Dneasy® Tissue Kit (Qiagen, Vienna, Austria). Following the instructions of the manufacturer, 25 mg of each organ (10 mg of spleen) were taken as starting material and the procedure was carried out as described in the Dneasy protocol for animal tissues. For elution of DNA in the final step, 200 μl of elution buffer were used. The samples were stored at −80 °C until processed.

For DNA extraction from in vitro propagated *Histomonas meleagridis* 100 μl of culture material were taken and frozen at −80 °C for two hours. Thereafter, the sample was thawed at room temperature and centrifuged at 3000 × *g* for 5 min. After removing the supernatant, the pellet was resuspended in 200 μl PBS and DNA extraction was performed using the DNeasy® Tissue Kit (Qiagen, Vienna, Austria) following the protocol for DNA extraction of cultured animal cells.

PCR was conducted as described recently [18]. Briefly, primer design was based on the small subunit rRNA sequence of *Histomonas meleagridis* published by Gerbod et al. [19]. Hot start procedures were used for PCR amplification using the "HotStarTaq Master Mix Kit" (Qiagen, Vienna, Austria). A 25-μl reaction mixture consisted of 12.5 μl HotStarTaq Master Mix, 1 μl of the specific forward primer (Hmf, 5′-GAA AGC ATC TAT CAA GTG GAA-3′) and 1 μl of the specific reverse primer for *Histomonas meleagridis* (Hmr, 5′-GAT CTT TTC AAA TTA GCT TTA AA-3′) (all primers were used in concentrations of 10 pmol/μl), 2.5 μl of DNA template and distilled water (added up to a volume of 25 μl). This reaction mixture was subjected to 40 cycles with an initial denaturation at 95 °C for 15 min, followed by heat denaturation at 94 °C for 30 s, primer annealing at 55 °C for 1 min and DNA extension at 72 °C for 1 min. Thereafter, the samples were maintained at 72 °C for 10 min for the final extension step. As negative controls tissue samples from uninfected control birds were used. They were included throughout the specimen preparation and PCR progress in order to control cross-contamination.

The specificity of the PCR was assessed by testing the primers with DNA from other protozoa (*Tetratrichomonas gallinarum*, *Trichomonas gallinae*, *Blastocystis* spp., *Eimeria tenella*, *Toxoplasma gondii*, *Cryptosporidia*, *Entamoeba invadens*, *Entamoeba ranarum*), fungi (*Aspergillus fumigatus*, *Candida albicans*), bacteria (*Staphylococcae*, *Streptococcae*, *E. coli*, *Clostridium perfringens*, *Campylobacter jejuni* NTCC 12145) and viruses (fowl adenovirus serotype 4, avian reovirus). No cross-reactivity could be observed with any of these microorganisms.

In order to evaluate the detection limit for the established PCR assay tenfold serial dilutions were prepared from the mono-eukaryotic culture of *Histomonas meleagridis* following DNA extraction. Primer set Hmf/Hmr detected *Histomonas meleagridis* DNA in a dilution containing only 10 histomonad organisms.

The dissemination of the 18S rRNA of *Histomonas meleagridis* in the selected tissues of the experimental animals was also evaluated by ISH. The method itself was conducted as described recently [20]. Briefly, a specific oligonucleotide probe called "HM" (5′-CCAACTACGTTAAAAATTATAAGAG TAGCTTTTCATT-3′) was used that hybridized with a segment of the 18S rRNA gene of *Histomonas meleagridis*. This oligonucleotide probe was labelled at the 3′ end with digoxigenin and used in a concentration of 20 ng per ml hybridization buffer. After over night incubation at 40 °C the slides with the tissue samples underwent three stringency washes using 2× standard saline citrate buffer (SSC), 1× SSC and 0.1× SSC in a decreasing order 10 min each at room temperature. The immunological detection of the hybrids was achieved using anti-digoxigenin-AP Fab. This reaction was visualized by the substances NBT (4-nitro blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolyl