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Short communication

Molecular assay for the detection of *Cochlosoma anatis* in house flies and turkey specimens by polymerase chain reaction

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Abstract

A 1520 bp region of *Cochlosoma anatis* mtDNA 16S gene was subjected to DNA sequencing and a 466 bp portion was compared with other protozoan 16S sequences to develop PCR primers specific for *C. anatis*. This PCR diagnostic method allowed identification of *C. anatis* from house flies, *Musca domestica* L., turkey gut, and fecal samples within 6 h after field-collected samples reached the laboratory. House flies detected carrying *C. anatis* using the diagnostic 374 bp amplicons represented the first record of this protozoan in house flies.

Keywords: PEMS; *Cochlosoma anatis*; PCR; Molecular diagnostics; House fly

1. Introduction

The flagellated protozoan, *Cochlosoma anatis* (Kotlan) (Retortamonadida: Cochlosomatidae), is often associated with enteritis in turkeys, *Meleagris gallopavo* L. (Cooper et al., 1995), and has been reported to cause poor weight gain and delayed tail-feather development in Muscovy ducks (Bollinger and Barker, 1996). They studied the effects of *C. anatis* on intestinal mucosal morphology in Muscovy ducks and found this parasite to have a detrimental effect on the digestive function of the intestinal mucosa. Lindsay et al. (1999) demonstrated that *C. anatis* could be transmitted to turkeys through oral inoculations.

C. anatis is generally diagnosed by visual identification of the protozoa using microscopic observation of scrapings from the intestines of poults. No molecular method was found that would provide rapid identification of the protozoa.

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We sequenced the mtDNA 16S rRNA gene from *C. anatis*, obtained from infected turkey intestines and developed a PCR marker specific for *C. anatis* for use to identify the flagellate protozoan from house flies, *Musca domestica* L., and turkey intestine and fecal samples.

2. Materials and methods

2.1. Protozoan strain and culture conditions

C. anatis infected intestinal tracts were procured from turkeys in North Carolina that were negative for Coronavirus antibodies based on indirect fluorescent antibody testing (Patel and Gonder, 1977). The parasite was identified based on the presence of an anterior adhesive disk, four anterior flagella, one dorsal flagellum, one recurrent flagellum and its characteristic motility pattern (Bermudez, 2003).

At the University of Missouri, the protozoa were maintained by inoculating 20 1-week-old poults with an intestinal inoculum prepared from a euthanized colony of 3-week-old *C. anatis* infected poults. This procedure resulted in the passage of the inoculum in poults every 2 weeks. Intestinal inoculums from the infected poults were transported to the University of Arkansas where successive passages were continued every 2 weeks.

Poults utilized at both university laboratories were acquired from a commercial hatchery where breeder flocks were monitored by the National Poultry Improvement plan and were negative for *Mycoplasma gallisepticum*, *Mycoplasma synovia* and *Mycoplasma meleagridis*. One-day-old female poults were brooded in stainless steel heated batteries in an environmentally controlled animal housing facility. The poults were given feed and water ad libitum. The University of Arkansas and the University of Missouri Animal Care and Use committees approved all animal use protocols.

2.2. DNA extraction

Total genomic DNA from house flies, turkey intestine and fecal samples infected with *C. anatis* was extracted using the Puregene DNA extraction kit (Gentra, Minneapolis, MN). Extracted DNA was resuspended in 50 μl Tris:EDTA pH 8.0 and frozen at −20 °C. To minimize protozoan contamination in the laboratory, all isolations were performed in a safety class II hood cabinet.

2.3. PCR and DNA sequencing

Extracted DNA from the turkey gut samples infected with *C. anatis* was subjected to PCR using the primers Euk 1900 (5′-AYYTGGTTGATYCTGCCY-3′) and Euk 1700 (5′-CBCCAGGTTCACCTAC-3′) from Gerbod et al. (2001), as per Szalanski et al. (2000). These primers amplify a 1520 bp region of the mtDNA 16S rRNA gene. PCR conditions consisted of an initial denaturation step of 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 3 min with a final extension step of 72 °C for 15 min. Amplified DNA from five *C. anatis* samples was purified, and concentrated using Microcon-PCR Filter Units (Millipore, Bedford, MA). Samples were sent to The University of Arkansas Medical Sciences DNA Sequencing Facility (Little Rock, AR) for direct sequencing in both directions using an ABI Prism 377 DNA sequencer.

2.4. Detection of C. anatis in poult intestines, feces and house flies

To test the molecular assay, fecal samples were taken as they were voided by six individually numbered laboratory-infected poults 2 weeks after they had been inoculated as previously described and the fecal deposits observed for the presence of *Cochlosoma* using a light-microscope. After finding *C. anatis*, the intestinal tracts were removed from six poults, and spatula-scraping samples from their intestines collected and DNA was extracted from all samples as previously described.

In addition, adult house flies, *Musca domestica* L., were collected from inside and in the area immediately surrounding the brooder and finishing facilities on a turkey farm located in northwest Arkansas where *C. anatis* had been diagnosed from intestine samples collected from poults on this farm during June 2002 (Dr. Alex Bermudez, Department of Veterinary Pathobiology, University of Missouri, Columbia, MO, personal communication). Flies were collected using aerial nets that had been previously autoclaved and exposed to UV light using a CL-1000 Crosslinker (UVP Inc., Upland, CA) to prevent PCR amplification

and possible contamination between sample collections. Flies captured in the aerial nets were killed by exposure to potassium cyanide using a killing jar immediately after field collection and placed in a ziplock bag, stored in a chest cooler and transported to the laboratory. In the laboratory, flies were identified to species and sex, placed in six dram glass vials, and stored at −80 °C. Voucher specimens, preserved in 95% ethanol, are maintained at the Arthropod Museum, Department of Entomology, University of Arkansas, Fayetteville, AR.

3. Results and discussion

No genetic variation was observed among the *C. anatis* DNA sequences, GenBank accession number AY704214. A 466 bp portion of the 16S rRNA DNA sequence was aligned with other protozoan sequences obtained from GenBank and examined for mismatches that reflected either substitutions or deletions. The mismatches were exploited to design primers that were unique to *C. anatis*. The amount of genetic divergence between *C. anatis* and the protozoan flagellates for the 466 bp region of the 16S rRNA gene obtained from GenBank (*Ditrichomonas honigbergi* U15505, *Kalotermes* gut symbiont AF215856, *Koruga bonita* AJ132467, *Monotrichomonas* sp. AF072905, *Pentatrichomonas hominis* AF124609, *Tetratrichomonas gallinarum* AF124608, *Trichomitopsis termopsidis* AF479642, and *Tritrichomonas foetus* AF466749) ranged from 8.2% to 11.2%. Two primers, one from each strand COCH-16S-F (5′-AAGGTTT-GTCATTTCAAAAT-3′) and COCH-16S-R (5′-TCT TCCTCCTGCTTAAATAA-3′) were designed. Based on the sequence, the expected sizes of the amplicon is 374 bp. Proper sized PCR products were obtained using conspecific DNA, whereas no product was obtained with template from the other species, i.e., no false positives were observed using known DNA. The *C. anatis* species-specific primers were tested for optimal annealing performance in a 41–59 °C temperature gradient with 2 °C intervals. The optimal annealing temperature for the FST specific primers was 43 °C. PCR conditions for the *C. anatis* specific primers consisted of an initial denaturation step of 94 °C for 5 min followed by 40 cycles of 94 °C for 45 s, 43 °C for 1 min and 72 °C for 1 min with a final extension step of 72 °C for 5 min. The *C. anatis* specific marker amplified successfully from *C. anatis* infected turkey gut and detected the flagellate protozoan from turkey feces (Fig. 1).

Using molecular diagnostics we identified *C. anatis* from the intestines of six poults and from six fecal samples collected from poults having laboratory infections. In addition, using molecular diagnostics, 8/18 (44%) adult house flies collected from the commercial turkey farm were found to contain

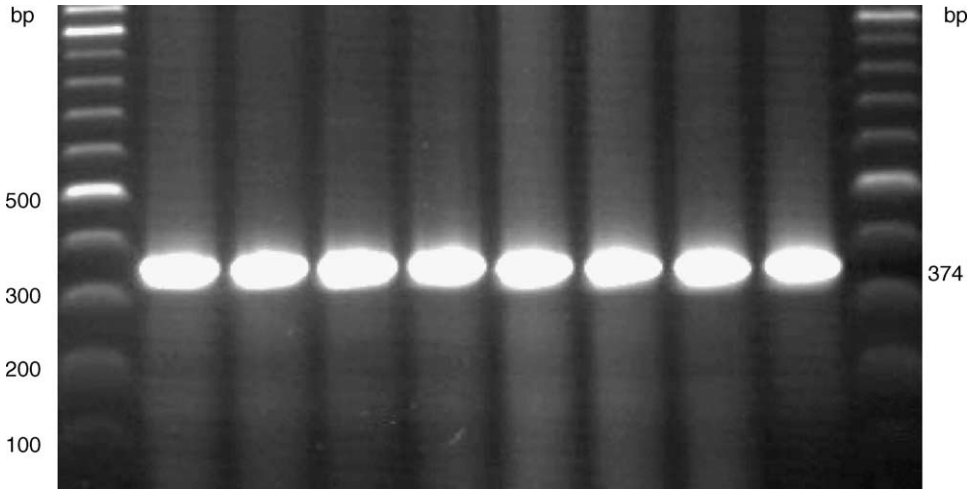

Fig. 1. PCR assay for *Cochlosoma anatis* (374 bp product), resolved on a 1% agarose gel. Each of the eight lanes of the gel represents identification of *C. anatis* in an individual house fly collected on a turkey farm.

C. anatis. This represented the first documented evidence that filth flies carry *C. anatis* in the poultry production environment and indicated that they may play a role in the dispersal and transmission of this protozoan parasite. Our polymerase chain reaction-based assay provided identification of pathogens by selective amplication of species or strain-specific regions of the organisms genome. The application of our method for the detection of *C. anatis* could help in determining the occurrence of the parasite in poultry facilities as well as for detection of the parasite in filth flies breeding in and around turkey production facilities that could serve as competent reservoirs.

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