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GIARDIA DUODENALIS CYSTS OF GENOTYPE A RECOVERED FROM CLAMS IN THE CHESAPEAKE BAY SUBESTUARY, RHODE RIVER

THADDEUS K. GRACZYK, R. C. ANDREW THOMPSON, RONALD FAYER, PETER ADAMS, UNA M. MORGAN, AND EARL J. LEWIS

Department of Molecular Microbiology and Immunology, and Department of Environmental Health Sciences, School of Hygiene and Public Health, Johns Hopkins University, Baltimore, Maryland; World Health Organization Collaborating Centre for the Molecular Epidemiology of Parasitic Infections and State Agricultural Biotechnology Centre, School of Veterinary Studies, Murdoch University, Murdoch, Perth, Western Australia, Australia; Immunity and Disease Resistance Laboratory, Livestock and Poultry Science Institute, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland; Cooperative Oxford Laboratory, Center for Coastal Environmental Health and Biomolecular Research, National Ocean Service, National Oceanic and Atmospheric Administration, U.S. Department of Commerce, Oxford, Maryland

Abstract. Filter-feeding molluscan shellfish can concentrate zoonotic and anthroponotic waterborne pathogens. Cysts of *Giardia* sp. were detected by immunofluorescent antibodies in tissues of the clams *Macoma balthica* and *M. mitchelli* from Rhode River, a Chesapeake Bay (Maryland) subestuary. Molecular tests identified the cysts as *Giardia duodenalis* Genotype A, the most common genotype recovered from humans. *Macoma* clams are burrowers in mud or sandy-mud substrata and preferentially feed on the surface sediment layer. Waterborne *Giardia* cysts settle rapidly to the bottom in slow-moving waters and contaminate the sediment. *Macoma* clams do not have economic value, but can serve as biologic indicators of sediment contamination with *Giardia* sp. cysts of public health importance. These clams can be used for sanitary assessment of water quality.

Bivalves can harbor environmentally derived pathogenic microorganisms as a result of filtering large volumes of water and concentrating the recovered particles.[1] Examination of bivalves can demonstrate water pollution even when water testing produces negative results.[2] For example, *Cryptosporidium parvum* oocysts had never been reported in Chesapeake Bay (Maryland) water until oysters *(Crassostrea virginica)* collected from that water were found to harbor this zoonotic parasite.[3] Similarly, *C. parvum* oocysts have been repeatedly recovered from the tissue of feral blue mussels *(Mytilus edulis)* from Sligo Bay (Ireland), but most of the water samples from the mussel collection sites were oocyst-negative.[4]

Giardia sp. are flagellated protozoan parasites of vertebrates; their infectious stage, the cyst, is transmitted via the fecal-oral route and frequently via water.[5] The genus *Giardia* has been divided into 3 morphologic types: *G. duodenalis* (= *lamblia, intestinalis*), *G. muris,* and *G. agilis.*[6] *Giardia duodenalis* and *G. muris* have been reported from mammals and birds.[5,7] In the wild, a wide variety of aquatic and semiaquatic mammals and birds[8] can be a source of waterborne *Giardia* cysts.[5,9,10]

Studies on giardiasis in a variety of birds suggest they may be zoonotic reservoirs;[7,9–12] however, these indications have not been proven.[13] As reviewed by Hopkins and others,[14] numerous studies demonstrated genetic and antigenic similarities among *Giardia* cyst isolates from humans and other mammalian hosts. Results of many cross-transmission experiments on the zoonotic potential of mammalian isolates of *Giardia* remain inconclusive.[15]

Identification of the genotype of *Giardia* directly from cysts appears to be the most reliable means to demonstrate zoonotic transmission.[14,16] Genotyping characterization of *G. duodenalis* from cyst samples originating from a human waterborne outbreak was identical to the genotype of cysts derived from beavers living in the same epidemic locality.[16] For studying the epidemiology of giardiasis, a polymerase chain reaction (PCR)–based method for genotyping *Giardia* isolates directly from cysts has been developed and successfully used to differentiate genotypes of the parasites originating from humans and dogs in Aboriginal communities in Western Australia.[14] This molecular identification does not require cultivation to increase the number of organisms and can be applied directly to the cysts recovered from environmental sources.[14]

Macoma balthica and *M. mitchelli* are common shellfish species throughout saline intertidal and subtidal zones of Chesapeake Bay.[17] These clams are rapid burrowers in mud or sandy-mud substrata. In addition to ingesting food particles from the surface layers of the sediment, they are able to filter seston (particulate matter) from overlaying waters.[17] Laboratory experiments demonstrated that waterborne *Giardia* cysts can be recovered by Asian freshwater clams (*Corbicula fluminea*), which are typical filter-feeders,[18] and internalized by the hemocytes of *Corbicula.*[19] Other than experimentally induced infections, there are no reports that *Giardia* cysts have been identified in feral freshwater or saltwater bivalve mollusks. The purposes of the present study were to determine if *M. balthica* or *M. mitchelli* clams collected in nature harbor *Giardia* cysts in their tissue and, if so, to use molecular typing techniques[14] to aid in determining the origin of these cysts, e.g., avian or mammalian hosts. The presence of *Giardia* cysts in the *Macoma* clam tissue would indicate fecal contamination of water and sediments.

MATERIALS AND METHODS

Clam collection and processing. *Macoma balthica* and *M. mitchelli* clams (n = 378 and n = 210, respectively) were collected in August 1998 from the central part of the Rhode River (38°52′N, 76°31′W), which is a subestuary of Chesapeake Bay (Maryland). Clams were harvested from sediment by sieving[20] and transported to the laboratory in a cooler. Clams of the same species were weighed (live weight) together, and the shell length of 30 randomly selected clams of each species was measured. Thirty of the largest *M. bal-*

thica clams (shell length = 25–38 mm) were opened[1] and hemolymph was aspirated from each (approximately 200 μl/clam). Pooled hemolymph was used to fill 8 chambers (approximately 750 μl capacity each) of a tissue culture glass slide (Nalgene; Nunc International, Naperville, IL), and the chambers were covered.

Macoma balthica and *M. mitchelli* clams were separately ground with a double volume (w/v) of phosphate-buffered saline (PBS) (pH 7.4) in a grinder designed for homogenization of soft tissue.[21] Homogenates of each species were separately sieved,[22] and the finest particle fractions (< 100 μm) were placed in conical-shaped sedimentation flasks (0.7 L capacity) and left overnight at 4°C. Sediment was aspirated from the flasks: 3.5 ml of *M. balthica* and 1.4 ml of *M. mitchelli.* These were pipetted into 5 and 2 chambers of the culture slide, respectively.

After incubation for 2 hr at 20°C, supernatant was aspirated from each chamber, plastic dividers were removed, and slides were air-dried and stained with immunofluorescent antibodies (IFA) (*MERIFLUOR™ Cryptosporidium/Giardia;* Meridian Diagnostic, Cincinnati, OH) as described previously.[3] The slides were examined with the aid of a fluorescence microscope[3] and *Giardia* sp. cysts were counted and measured (n = 30).

The remaining sediments from each clam species were purified over a $CsCl_2$ gradient by centrifugation.[23] All $CsCl_2$ phase fractions were processed by the cellulose acetate membrane (CAM)-filter dissolution method.[24] The resulting pellets were separately resuspended in 500 μl of PBS: 50-μl aliquots were tested by IFA, and the remaining material was used for molecular analyses.

Genotyping of *Giardia* sp. cysts. Molecular characterization of *Giardia* isolates was carried out by PCR amplification and sequencing of a 292-basepair (bp) region near the 5′ end of the small subunit-rRNA gene (SSU-rRNA) as described previously.[14]

Isolation of DNA. Approximately 100–200 μl of *Giardia* sp. cyst suspension was added to a clean 1.5-ml Eppendorf tube (VWR Scientific, Bridgeport, NJ) with 80 μl of tissue lysis buffer (Qiagen, Hilden, Germany) and boiled for 10 min; 180 μl of AL buffer (Qiagen), followed by 10 μl of glassmilk (Bio-Rad Laboratories, Hercules, CA) was added to cyst suspension, which was vortexed well and incubated at 72°C for 10 min. Samples were centrifuged at 14,000 rpm for 1 min in an Eppendorf Centrifuge 5417C (VWR Scientific) and the supernatant was discarded. The pellet was washed with 700 μl of AW wash buffer (Qiagen) by vortexing well. The suspension was centrifuged again at 14,000 rpm for 1 min and the supernatant was discarded. The wash step was repeated and the pellet was dried in a vacuum desiccator for 5–10 min. The pellet was resuspended in 20 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA) and incubated at 72°C for 5 min. Samples were centrifuged at 14,000 rpm for 2 min and the supernatant containing the DNA was transferred into a clean tube.

Amplification by the PCR and sequencing. The primers used to perform the PCR on the isolated DNA were the forward primer (1–18) RH11 [5′-CATCCGGTCGATCC TGCC-3′] and the reverse primer (268–292) RH4 [5′-AGTCGAACCCTGATTCTCCGCCAGG-3′], which are both complementary to a 292-bp region of the 5′ end of the 16S-rRNA gene.[14] The PCR amplification was performed in 50-μl volumes with the final mixture containing 1–10 ng of DNA, 5% dimethyl sulfoxide (DMSO), 12.5 pmol of each primer, 2.2 units of *Tth* plus DNA polymerase (Biotech International, Perth, Australia), 2 units of *Taq* extender (Stratagene, La Jolla, CA), 1 mM of each dNTP, 2 mM $MgCl_2$, 67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 0.2 mg/ml of gelatin, and water. Preparations were heated to 96°C for 2 min followed by 50 cycles of 96°C for 20 sec, 59°C for 20 sec, 72°C for 30 sec, and 1 cycle of 72°C for 7 min using a GeneAmp PCR System 2400 thermocycler (Perkin-Elmer, Foster City, CA). Templates were purified from excess dNTPs and primers using PCR spin columns (Qiagen). Sequencing reactions were performed using an ABI Prism Dye Terminator Cycle Sequencing Core kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions, except that 5% DMSO was included in the reaction mixture, and an annealing temperature of 59°C was used. Sequence profiles were analyzed using SeqEd, version 0.3 (Applied Biosystems).

Statistical analysis. Statistical analysis was carried out with Statistix 4.1 (Analytical Software, St. Paul, MN). Analysis of variance (ANOVA) was used to assess significance of the differences between variables. Mean values were associated with the SD, and statistical significance was considered to be a P value < 0.05.

RESULTS

Clam collection and processing. The total live weights of *M. balthica* and *M. mitchelli* clams were 1,917 g and 481 g, respectively. The range of shell lengths of *M. balthica* and *M. mitchelli* was 21–38 mm (mean ± SD = 33.6 ± 4.5 mm) and 16–19 mm (mean ± SD = 18.1 ± 1.0 mm), respectively. *Giardia* sp. cysts were detected in all 8 hemolymph and 5 tissue homogenate samples of *M. balthica;* the number of cysts varied from 3 to 8 cyst/well (mean = 5.6) and from 12 to 19 (mean = 16.2 cysts/well), respectively. Five and 12 *Giardia* sp. cysts were detected in 2 wells filled with homogenized tissue of *M. mitchelli* clams. *Giardia* sp. cysts detected in the hemolymph and homogenized clam tissue fluoresced bright green of a similar intensity as that of the human isolate of *Giardia* cysts in the positive control of the *MERIFLUOR™*test kit. Clam-recovered *Giardia* sp. cysts were oval and within the range of 11.7–14.2 μm (mean ± SD = 13.2 ± 0.9 μm) by 7.4–11.0 μm (mean ± SD = 9.3 ± 1.2 μm). The difference in cyst sizes recovered from the 2 clam species were not significant ($F = 5.92$, $P < 0.01$, by ANOVA). Of tissue from 378 *M. balthica* and 210 *M. mitchelli* clams homogenized, sieved, sedimented, and purified over a $CsCl_2$ gradient, approximately 31 and 22 *Giardia* sp. cysts per gram were recovered, respectively.

Cryptosporidium sp. oocysts were not detected in any of the samples.

Molecular characterization. Sequence analysis of the 292-bp SSU-rDNA PCR product showed that both isolates of *Giardia* sp. cysts conformed to that of the Group A genotype.

DISCUSSION

Current data on *Giardia* do not support the traditional view that numerous, highly host-specific *Giardia* species exist,[25] but rather indicate the existence of a limited number of species. Of these, *G. duodenalis* exhibits considerable intraspecific variability with a number of genotypes, some of which are not rigidly host-specific.[5,15] Therefore, genotypic identification of a genotype of cysts recovered from environmental source(s) is epidemiologically important to determine their zoonotic potential, and a plausible source of environmental contamination. In the present study, the morphometric analysis of *Giardia* cysts recovered from 2 species of *Macoma* clams from the same location showed no differences between the isolates. These cysts belonged to a single genotype (Genotype A) of *G. duodenalis,* the most common genotype infecting mammals, including humans.[26] Based on these results, we conclude that water and the sediments of that Chesapeake Bay site must have been contaminated with feces of mammalian origin that contained *G. duodenalis* cysts of public health importance.

The area from which *Macoma* clams were collected is a wildlife refuge with a high activity of beavers, muskrats, river otters, and deer. Beavers *(Castor canadiensis)* and muskrats (*Ondatra zibethicus)* have long been considered significant reservoirs for this parasite, and it has been demonstrated that some *Giardia* sp. cysts recovered from drinking water originated from these mammals.[16,27] Thus, the wildlife in the Rhode River area could be the parasite source. On the other hand, wastewater origin of the cysts cannot be excluded because there is a direct wastewater discharge into the Rhode River by the Mayo Large Communal Water Reclamation Facility.[28] In addition, based on an elevated fecal coliform level in the Rhode River, this area is classified by the State of Maryland as conditionally approved for harvesting of shellfish.[29]

Giardia cysts can remain viable in surface water for approximately 2 months.[30] In the water, these cysts gravitate to the bottom of the reservoir and are more easily found in the sediment than in the water.[31] Also, sedimentation is the most efficient procedure for the removal of *Giardia* cysts during treatment of drinking water.[32] A long-term (more than 14 months) ecologic investigation demonstrated that *Giardia* sp. cysts originating from beaver and muskrat colonies settled rapidly to the bottom of slow-moving water reservoirs and contaminated the sediment.[27] *Macoma* clams preferentially feed on detritus or particulates from sediments.[17] They have flexible siphons of unequal length. The longer one, the inhalant siphon, is protruded from the burrow and sweeps across the soft bottom sediment vacuuming up particles.[17] Thus, it is more likely that *Giardia* cysts that have settled to the bottom will be ingested by clams, rather than suspended throughout the water column. This may explain why, despite the extensive ecologic surveys of Chesapeake Bay for *Cryptosporidium* and *Giardia,* the latter parasite has never been found in eastern oysters[3] or bent mussels,[33] which are exclusive filter feeders not ingesting sediments.[17]

Cryptosporidium parvum oocysts have been recovered from other feral shellfish of Chesapeake Bay, i.e., oysters[3] and bent mussels.[33] However, in contrast to *Giardia* cysts, sedimentation *of Cryptosporidium* oocysts in the water is extremely slow,[32] and under most natural conditions few oocysts are deposited in the sediments.

The present study demonstrated a concentration of *G. duodenalis* in *M. balthica* and *M. mitchelli* of approximately 31 and 22 cysts/g, respectively. Experimental spiking of homogenized tissue of Asian freshwater clams (*C. fluminea*) (similar in size to *Macoma* clams) with *Giardia* cysts demonstrated that the recovery efficiency of cysts does not usually exceed 50%.[18] Thus, the actual numbers of *G. duodenalis* cysts retained by the *Macoma* clams could be twice as high.

Previous ecologic studies demonstrated human waterborne pathogens in the Rhode River[34,35] and high levels of biologically derived nitrogen in the sediments.[36] *Vibrio parahaemolyticus* was recovered from Rhode River sediment and water,[34] and *Clostridium botulinum* was found in the water.[35] The central portion of the Rhode River had fewer coliforms, fecal coliforms, and fecal streptococci than Muddy Creek (a marsh receiving pasture runoff) and Caddle Creek (a heavily populated area);[35] both creeks empty into the Rhode River. In addition to these findings, the present study reports the presence of *G. duodenalis* cysts in the Rhode River.

Economically important shellfish, such as oysters, which are harvested commercially and preferentially consumed raw, can be of public health importance if contaminated with human waterborne pathogens.[3,37,38] Shellfish that do not have an apparent economic value may serve as indicators in monitoring aquatic environments for pollution with zoonotic and anthroponotic pathogens, such as *Giardia or Cryptosporidium.* The ability of *Macoma* clams to recover cysts of *G. duodenalis* from the environment indicate their applicability for the biological monitoring of sediment contamination with this human pathogen. *Macoma* clams, which are common throughout Chesapeake Bay tributaries,[17] represent a convenient organism for biological monitoring of sediment contamination because they have no apparent economical value, are collectible throughout the year, and have a relatively small size (maximum shell length = 4 cm).[17]

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Authors' addresses: Thaddeus K. Graczyk, Department of Molecular Microbiology and Immunology, Johns Hopkins University, School of Hygiene and Public Health, Baltimore, MD 21205. R. C. Andrew Thompson, Peter Adams, and Una M. Morgan, World Health Organization Collaborating Centre for the Molecular Epidemiology of Parasitic Infections and State Agricultural Biotechnology Centre, School of Veterinary Studies, Murdoch University, Murdoch, Perth 6150, Western Australia. Ronald Fayer, Immunity and Disease Resistance Laboratory, Livestock and Poultry Science Institute, Agricultural Research Services, U.S. Department of Agriculture, Beltsville, MD 20705. Earl J. Lewis, Cooperative Oxford Laboratory, Center for Coastal Environmental Health and Biomolecular Research, National Ocean Service, National Oceanic and Atmospheric Administration, U.S. Department of Commerce, Oxford, MD 21654.

Reprint requests: Thaddeus K. Graczyk, Department of Molecular Microbiology and Immunology, Johns Hopkins University, School of Hygiene and Public Health, Baltimore, MD 21205.

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