JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2010, p. 2988–2990 Vol. 48, No. 8
0095-1137/10/$12.00 doi:10.1128/JCM.00013-10

Molecular Identification of *Entamoeba* spp. in Captive Nonhuman Primates▽†

B. Levecke,[1,2]* Leentje Dreesen,[1] Pierre Dorny,[1,3] Jaco J. Verweij,[4] Francis Vercammen,[2] Stijn Casaert,[1] Jozef Vercruysse,[1] and Peter Geldhof[1]

Laboratory of Parasitology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium[1]; Centre for Research and Conservation, Royal Zoological Society of Antwerp, Koningin Astridplein 26, B-2018 Antwerp, Belgium[2]; Department of Animal Health, Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerp, Belgium[3]; and Department of Parasitology, Leiden University Medical Center, 14 Postbus 9600, 2300 RC Leiden, Netherlands[4]

Received 4 January 2010/Returned for modification 27 May 2010/Accepted 14 June 2010

This study describes the molecular identification of 520 *Entamoeba*-positive fecal samples from a large and diverse population of captive nonhuman primates (NHP). The results revealed the presence of *Entamoeba histolytica* (NHP variant only), *E. dispar*, *E. moshkovskii*, *E. hartmanni*, *E. coli*, and *E. polecki*-like organisms.

Various *Entamoeba* species are frequently found in the stools of both captive (15, 22) and wild (5, 7) nonhuman primates (NHP). Although the majority of these *Entamoeba* spp. are considered to be harmless, care should be taken when *E. histolytica*, the causative agents of amoebiasis, is involved. Infection with this gastrointestinal parasite in NHP may cause hemorrhagic dysentery (6, 28) and extraintestinal pathologies (e.g., liver abscesses) and death (12, 16). Moreover, amoebiasis is of major concern in public health, resulting in similar pathologies in humans and causing up to 100,000 deaths worldwide each year (19). Currently, little is known about the occurrence of *E. histolytica* in NHP, and the role of these animals as a potential reservoir for zoonotic transmission remains unclear. Most of the previous studies were based on the detection of cysts or trophozoites in stools by using light microscopy. However, differentiation between *E. histolytica* and other *Entamoeba* spp. (such as *E. coli*, *E. hartmanni*, and *E. polecki*-like organisms) based on morphological features is difficult (8, 27) and when *E. dispar* or *E. moshkovskii* is involved, it can even be impossible (4). For this purpose, molecular methods are more appropriate. Furthermore, recent molecular analyses of *E. histolytica* indicate genetic differences between human and NHP isolates (21, 23, 24). Although these differences may contribute to the elucidation of zoonotic transmission pathways, little is known about the distribution of these *E. histolytica* variants in both humans and NHP. Therefore, the objective of the present study was to identify the *Entamoeba* spp. in a large and diverse population of captive NHP, including differentiation between the human and NHP variants of *E. histolytica*.

A total of 520 stool samples containing *Entamoeba* cysts were selected for further molecular identification. These samples were obtained from previous epidemiological surveys (10; unpublished data) and were stored at −20°C. The animals were housed in nine zoological gardens and one sanctuary in Belgium and the Netherlands, representing 58 NHP groups belonging to 36 animal species (see Table S1 in the supplemental material). None of the animals showed clinical signs associated with gastrointestinal disorders. DNA was extracted using the QIAamp stool minikit according the instructions of the manufacturer (Qiagen) and the adaptations described previously (9). The identification of *E. histolytica*, *E. dispar*, *E. moshkovskii*, *E. hartmanii*, *E. coli*, and *E. polecki*-like organisms was based largely on a previously described PCR-reverse line hybridization blot (PCR-RLHB) protocol targeting the small-subunit rRNA gene (26). This assay was preferred since it allows the simultaneous detection of various *Entamoeba* species. The amplification reactions were performed in a volume of 25 μl containing 2.5 μl DNA, 0.5 μl of each primer (10 μM), 1 μl $MgCl_2$ (25 mM), 5 μl GoTaq Flexi buffer, 14.875 μl PCR-grade H_2O, and 0.125 μl GoTaq Flexi DNA polymerase. For *E. histolytica*, a novel probe (5′-YAT TRA ATR AAT TGG CCA TTT TGT A-3′) was designed based on the gene sequences of the human variant (GenBank accession number X64142) and the NHP variant (GenBank accession numbers AB197936 and AB282657) to ensure the detection of both variants. In each PCR-RLHB run, control DNA samples from *E. histolytica* (both variants), *E. dispar*, *E. moshkovskii*, *E. hartmanni*, *E. coli*, and *E. polecki*-like organisms were included. Samples showing hybridization with the *E. histolytica* probe were retained for additional differentiation between the human and the NHP variants by using novel variant-specific reverse primers (human variant primer, 5′-CAT TTC TAG AAA CTT TAC TTA CAT-3′; NHP variant primer, 5′-CAT TTC TAG AAA CTT TAC TTA TGC-3′) designed from sequences with the GenBank accession numbers mentioned above. The amplification conditions remained unchanged. In each PCR run, control DNA samples from both the human variant and the NHP variant of *E. histolytica* were included. PCR products were run on agarose gels, stained with ethidium bromide, and detected upon UV transillumination. Samples reacting only with the general *Entamoeba* probe and not with any of the species-specific probes were retained for further sequence analyses. To this end, the PCR preceeding the RLHB assay was repeated with unlabeled primers. The obtained PCR products were purified with QIAquick purification columns (Qiagen, Germany) and cloned into the pGEM-T Easy vector ac-

* Corresponding author. Mailing address: Laboratory of Parasitology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium. Phone: 32-9-264 74 04. Fax: 32-9-264 74 96. E-mail: Bruno.Levecke@Ugent.be.

† Supplemental material for this article may be found at http://jcm.asm.org/.

▽ Published ahead of print on 23 June 2010.

TABLE 1. Numbers of mono- and mixed infections with *Entamoeba* spp. in 372 samples based on a PCR-RLHB protocol targeting the small-subunit rRNA gene

Infecting organism(s)	No. (%) of samples
Organisms in monoinfections	109 (29.3)
E. histolytica	30 (8.1)
E. hartmanni	36 (9.7)
E. coli	12 (3.2)
E. polecki-like organisms	31 (8.3)
Organisms in mixed infections	193 (51.9)
E. histolytica + *E. hartmanni*	27 (7.3)
E. histolytica + *E. coli*	1 (0.3)
E. histolytica + *E. polecki*-like organisms	30 (8.1)
E. hartmanni + *E. coli*	28 (7.5)
E. hartmanni + *E. polecki*-like organisms	28 (7.5)
E. coli + *E. polecki*-like organisms	2 (0.5)
E. histolytica + *E. coli* + *E. polecki*-like organisms	3 (0.8)
E. histolytica + *E. hartmanni* + *E. coli*	3 (0.8)
E. histolytica + *E. hartmanni* + *E. polecki*-like organisms	36 (9.7)
E. dispar + *E. hartmanni* + *E. coli*	4 (1.1)
E. dispar + *E. hartmanni* + *E. polecki*-like organisms	3 (0.8)
E. moshkovskii + *E. hartmanni* + *E. coli*	2 (0.5)
E. moskovskii + *E. hartmanni* + *E. polecki*-like organisms	1 (0.3)
E. hartmanni + *E. coli* + *E. polecki*-like organisms	15 (4.0)
E. histolytica + *E. hartmanni* + *E. coli* + *E. polecki*-like organisms	4 (1.1)
E. dispar + *E. hartmanni* + *E. coli* + *E. polecki*-like organisms	2 (0.5)
E. moshkovskii + *E. hartmanni* + *E. coli* + *E. polecki*-like organisms	4 (1.1)
Unidentified *Entamoeba* organisms	70 (18.9)

cording to the instructions of the manufacturer (Promega, Madison, WI). Clones containing the expected amplicon of approximately 550 bp were sequenced using the BigDye Terminator kit (Applied Biosystems). Sequence reactions were analyzed with an ABI-3730xl sequencer (Applied Biosystems), and sequences were assembled using Seqman II (DNAstar, Madison, WI).

The RLHB analysis revealed the presence of *Entamoeba* DNA in 372 (71.5%) of 520 samples. The distribution of the different *Entamoeba* spp. within these 372 samples is described in Table 1. *E. hartmanni* (present in 51.9% of samples) was the most prevalent species, followed by *E. polecki*-like organisms (in 42.7% of samples), *E. histolytica* (in 36.0% of samples), and *E. coli* (in 21.5% of samples). *E. dispar* (present in 2.4% of samples) and *E. moskovskii* (present in 1.9% of samples) were found in only a small number of samples. Most samples (51.9%) carried mixed infections. A large proportion of the samples (18.8%) hybridized with the general *Entamoeba* probe but could not be assigned to any of the known *Entamoeba* spp. The *E. histolytica* variant-specific PCR revealed solely the NHP variant in 124 of the 132 *E. histolytica*-positive samples. For the remaining 8 samples, no amplification was found in either PCR protocol.

From the 70 samples which could not be assigned to known *Entamoeba* spp., 20 samples originating from 20 different NHP groups were withheld for sequencing, resulting in 21 clones (for one sample, two clones were analyzed). Twelve clones could be assigned to one of the known *Entamoeba* spp., including the *E. histolytica* NHP variant (5), *E. dispar* (2), *E. hartmanni* (3), or *E. coli* (2). Four clones did not reveal homology with *Entamoeba* spp. Instead, homology was found to DNA sequences from *Gregarina* (GenBank accession no. FJ459742) and *Saccharomyces* (GenBank accession no. FN393078) species, humans (GenBank accession no. CT476837), and *Galactomyces* species (GenBank accession no. X69842). The remaining four clones showed homology to *Entamoeba* spp., but the sequences did not match completely with those from one of the known *Entamoeba* spp. (*E. terrapinae* [GenBank accession no. AF149910], *E. insolita* [GenBank accession no. AF149909], *E. invadens* [GenBank accession no. AF149905], *E. ranarum* [GenBank accession no. AF149908], *E. equi* [GenBank accession no. DQ286371], *E. ecuadoriensis* [GenBank accession no. DQ286373], and *E. struthionis* [GenBank accession no. AJ566411]). All four sequences showed the least homology (74.2% to 75.2%) to sequences from *E. invadens*. The highest level of homology found (87.4% to 88.7%) was to sequences from *E. moshkovskii*. The sequences of these four clones were submitted to GenBank under accession no. GU437823 (for clone JL70 from a Javan lutung), GU437824 (for clone JL2399 from a Javan lutung), GU437825 (for clone MG107 from a mantled guereza), and GU437826 (for clone NPGL93 from a Northern plains gray langur).

To our knowledge, this is the first study that describes the molecular identification of *Entamoeba* isolates from a large and diverse population of captive NHP based on a PCR-RLHB protocol and an *E. histolytica* variant-specific PCR approach. The results confirm the presence of the *Entamoeba* spp. described previously but also suggest NHP (the owl-faced monkey, Javan lutung, and Northern plains gray langur) as novel host species for the free-living *E. moshkovskii*. Until now, *E. moshkovskii* had been detected only in samples from sewage and humans (1, 3). *E. hartmanni* and *E. polecki*-like organisms were the most prevalent; approximately half of the samples contained one of these *Entamoeba* species. *E. polecki*-like organisms are considered to be harmless for NHP, but their presence warrants caution. The *E. polecki*-like organisms may cause postmortem pathologies mimicking those caused by *E. histolytica* (29), consequently hindering an accurate diagnosis.

E. histolytica DNA was detected in a large proportion (36.0%) of the samples, originating from 11 of 58 NHP species examined. Five of these species (the vervet monkey, crab-eating macaque, rhesus monkey, Hamadryas baboon, and chimpanzee) (12, 25) have been reported previously to be hosts of *E. histolytica*. New host species identified in the present study are the tantalus monkey, greater spot-nosed monkey, Sunda pig-tailed macaque, olive baboon, and Bornean orangutan. Although previously found in other studies, *E. histolytica* was not found in the patas monkey (2, 12), mandrill (13, 28), mantled guereza (11, 20), and Western gorilla (18). The occurrence of *E. histolytica* is surprisingly high and was limited mainly to Old World monkeys. At the time of sampling, clinical symptoms were absent in all these animals. However, due to the study design, the clinical importance of these infections could not be investigated in more depth. Among the isolates of

E. histolytica, only the NHP variant was identified. This finding supports the hypothesis that this variant is restricted to NHP (20, 23, 24) but is in contrast with the results of a recent study in the Philippines, where the human variant was found in NHP (17). Although this would imply anthropozoonotic transmission, the distribution of both variants among humans and NHP remains largely unknown, underlining the importance of using variant-specific PCR approaches in future epidemiological surveys.

Sequence analyses revealed the presence of four novel *Entamoeba*-like sequences, which could not be assigned to any of the previously described *Entamoeba* spp. Studies analyzing additional genes, morphological features (trophozoites and cysts), and virulence are needed to determine their phylogenetic position within the genus *Entamoeba*.

Finally, this study underlines the need for improving detection techniques, as the presence of *Entamoeba* based on microscopic examination was not confirmed by PCR for a considerable proportion of the samples (38.5%). Although an initial microscopic misclassification cannot be ruled out, an interlaboratory comparison of a subset of the samples examined indicated moderate agreement in the microscopic findings (data not shown). Therefore, the most important factor contributing to this discrepancy for both techniques is probably the inhibition of the PCR by fecal components, a well-known problem in the detection of pathogens in feces (14). Moreover, the previously described primers for the detection of *Entamoeba* spp. are not as specific as previously assumed, which can be explained by small sequence differences in the primer regions.

In conclusion, this study confirms the presence of previously described *Entamoeba* spp. in NHP and is the first report of *E. moshkovskii* in these animals. The results also indicate that *E. histolytica* (exclusively the NHP variant) is prevalent in captive NHP, supporting the host specificity of this NHP variant of *E. histolytica*. However, studies using variant-specific PCRs are needed to elucidate the epidemiology of both variants of *E. histolytica*. Finally, this study reveals the presence of novel *Entamoeba*-like sequences, which warrants further attention.

This work was funded by the Centre for Research and Conservation of the Royal Zoological Society of Antwerp. P.G. is a postdoctoral fellow of the Fund for Scientific Research-Flanders (Belgium; F.W.O.-Vlaanderen).

We also thank the veterinarians and animal caretakers of the sanctuary for exotic animals AAP (Netherlands), Amsterdam Zoo (Netherlands), Apenheul Primate Park (Netherlands), GAIA Park Zoo (Netherlands), Rotterdam Zoo (Netherlands), Safari Park Beekse Bergen (Netherlands), Antwerp Zoo (Belgium), Animal Park Planckendael (Belgium), Olmen Zoo (Belgium), and Park Paradisio (Belgium) for providing the samples.

REFERENCES

1. **Ali, I. K. M., M. B. Hossain, S. Roy, P. F. Ayeh-Kumi, W. A. Petri, Jr., R. Haque, and C. G. Clark.** 2003. *Entamoeba moshkovskii* infections in children in Bangladesh. Emerg. Infect. Dis. **9:**580–584.
2. **Beaver, P. C., J. L. Blanchard, and H. R. Seibold.** 1988. Invasive amebiasis in naturally infected New World and Old World monkeys with and without clinical disease. Am. J. Trop. Med. Hyg. **39:**343–352.
3. **Clark, C. G., and L. S. Diamond.** 1991. The Laredo strain and other '*Entamoeba histolytica*-like' amoebae are *Entamoeba moshkovskii*. Mol. Biochem. Parasitol. **46:**11–18.
4. **Diamond, L. S., and C. G. Clark.** 1993. A redescription of *Entamoeba histolytica* Schaudinn, 1903 (emended Walker, 1911) separating it from *Entamoeba dispar* Brumpt, 1925. J. Eukaryot. Microbiol. **40:**340–344.
5. **Gillespie, T. R., E. C. Greiner, and C. A. Chapman.** 2005. Gastrointestinal parasites of the colobus monkeys of Uganda. J. Parasitol. **91:**569–573.
6. **Haq, A., A. Sharma, S. Ahmad, H. M. Khan, and N. Khan.** 1985. Experimental infection of rhesus monkeys with *Entamoeba histolytica* mimics human infection. Lab. Anim. Sci. **35:**481–484.
7. **Karere, G. M., and E. Munene.** 2002. Some gastro-intestinal tract parasites in wild De Brazza's monkeys (*Cercopithecus neglectus*) in Kenya. Vet. Parasitol. **110:**153–157.
8. **Kebede, A., J. J. Verweij, W. Dorigo-Zetsma, E. Sanders, T. Messele, L. van Lieshout, B. Petros, and A. Polderman.** 2003. Overdiagnosis of amoebiasis in the absence of *Entamoeba histolytica* among patients presenting with diarrhoea in Wonji and Akaki, Ethiopia. Trans. R. Soc. Trop. Med. Hyg. **97:**305–307.
9. **Levecke, B., P. Geldhof, E. Claerebout, P. Dorny, F. Vercammen, S. M. Cacciò, J. Vercruysse, and T. Geurden.** 2009. Molecular characterisation of *Giardia duodenalis* in captive nonhuman primates reveals mixed assemblage A and B infections and novel polymorphisms. Int. J. Parasitol. **139:**1595–1601.
10. **Levecke, B., P. Dorny, T. Geurden, F. Vercammen, and J. Vercruysse.** 2007. Gastrointestinal protozoa in nonhuman primates of four zoological gardens in Belgium. Vet. Parasitol. **148:**236–246.
11. **Loomis, M. R., J. O. Britt, Jr., A. P. Gendron, H. J. Holshuh, and E. B. Howard.** 1983. Hepatic and gastric amebiasis in black and white colobus monkeys. J. Am. Vet. Med. Assoc. **183:**1188–1191.
12. **Márquez-Monter, H., R. Fuentes-Orozco, I. Correa-Lemus, and I. Becker.** 1991. Invasive amebiasis in a spider monkey (*Ateles geoffroyi*). Case report and a short review of the literature of amebiasis in nonhuman primates. Arch. Invest. Med. (Mex.) **21:**75–78.
13. **Mätz-Rensing, K., A. Floto, F.-J. Kaup, and K. Schaller.** 2004. Gastric amebiasis due to *Entamoeba histolytica* infection in a mandrill (*Mandrillus sphinx*), p. 325–327. Proc. Eur. Assoc. Zoo Wildl. Vet. 5th Sci. Meet., 19 to 23 May 2004, Ebeltoft, Denmark. European Association of Zoo and Wildlife Veterinarians, Liebefeld-Berne, Switzerland.
14. **Monteiro, L., D. Bonnemaison, A. Vekris, K. G. Petry, J. Bonnet, R. Vidal, J. Cabrita, and F. Mégraud.** 1997. Complex polysaccharides as PCR inhibitors in feces: *Helicobacter pylori* model. J. Clin. Microbiol. **35:**995–998.
15. **Munene, E., M. Otsyula, D. A. N. Mbaabu, W. T. Mutahi, and S. M. K. Muriuki.** 1998. Helminth and protozoan gastrointestinal tract (GIT) parasites in captive and wild-trapped African nonhuman primates. Vet. Parasitol. **78:**195–201.
16. **Pang, V. F., C. C. Chang, and W. F. Chang.** 1993. Concurrent gastric and hepatic amebiasis in a dusky leaf monkey (*Presbytis obscurus*). J. Zoo Wildl. Med. **24:**204–207.
17. **Rivera, W. L., J. A. D. L. Yason, and D. E. V. Adao.** 2010. *Entamoeba histolytica* and *E. dispar* infections in captive macaques (*Macaca fascicularis*) in the Philippines. Primates **51:**69–74.
18. **Sleeman, J. M., L. L. Meader, A. B. Mudakikwa, J. W. Foster, and S. Patton.** 2000. Gastrointestinal parasites of mountain gorillas (*Gorilla gorilla beringei*) in the Parc National des Volcans, Rwanda. J. Zoo Wildl. Med. **31:**322–328.
19. **Stauffer, W., and I. J. Ravdin.** 2003. *Entamoeba histolytica*: an update. Curr. Opin. Infect. Dis. **16:**479–485.
20. **Suzuki, J., S. Kobayashi, R. Murata, H. Tajima, F. Hashizaki, Y. Yanagawa, and T. Takeuchi.** 2008. A survey of amoebic infections and differentiation of an *Entamoeba histolytica*-like variant (JSK2004) in nonhuman primates by a multiplex polymerase chain reaction. J. Zoo Wildl. Med. **39:**370–379.
21. **Suzuki, J., S. Kobayashi, R. Murata, Y. Yanagawa, and T. Takeuchi.** 2007. Profiles of a pathogenic *Entamoeba histolytica*-like variant with variations in the nucleotide sequence of the small subunit ribosomal RNA isolated from a primate (De Brazza's guenon). J. Zoo Wildl. Med. **38:**471–474.
22. **Tachibana, H., X.-J. Cheng, S. Kobayashi, N. Matsubayashi, S. Gotoh, and K. Matsubayashi.** 2001. High prevalence of infection with *Entamoeba dispar*, but not *E. histolytica*, in captive macaques. Parasitol. Res. **87:**14–17.
23. **Tachibana, H., T. Yanagi, K. Pandey, X.-J. Cheng, S. Kobayashi, J. B. Sherchand, and H. Kanbara.** 2007. An *Entamoeba* sp. strain from rhesus monkey is virulent but genetically different from *Entamoeba histolytica.* Mol. Biochem. Parasitol. **153:**107–114.
24. **Takano, J.-I., T. Narita, H. Tachibana, K. Terao, and K. Fujimoto.** 2007. Comparison of *Entamoeba histolytica* DNA isolated from a cynomolgus monkey with human isolates. Parasitol. Res. **101:**539–546.
25. **Takano, J.-I., T. Narita, H. Tachibana, T. Shimizu, H. Komatsubara, K. Terao, and K. Fujimoto.** 2005. *Entamoeba histolytica* and *E. dispar* infections in cynomolgus monkeys imported into Japan for research. Parasitol. Res. **97:**255–257.
26. **Verweij, J. J., D. Laeijendecker, E. A. T. Brienen, L. van Lieshout, and A. M. Polderman.** 2003. Detection and identification of *Entamoeba* species in stool samples by a reverse line hybridization assay. J. Clin. Microbiol. **41:**5041–5045.
27. **Verweij, J. J., A. M. Polderman, and C. G. Clark.** 2001. Genetic variation among human isolates of uninucleated cyst-producing *Entamoeba* species. J. Clin. Microbiol. **39:**1644–1646.
28. **Verweij, J. J., J. Vermeer, E. A. T. Brienen, C. Blotkamp, D. Laeijendecker, L. van Lieshout, and A. M. Polderman.** 2003. *Entamoeba histolytica* infections in captive primates. Parasitol. Res. **90:**100–103.
29. **Vogel, P., G. Zaucha, D. Goodwin, K. Kuehl, and D. Fritz.** 1996. Rapid postmortem invasion of cecal mucosa of macaques by nonpathogenic *Entamoeba chattoni*. Am. J. Trop. Med. Hyg. **55:**595–602.