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Hiroshi Tachibana · Xun-Jia Cheng · Seiki Kobayashi
Yukitoki Fujita · Toshifumi Udono

Entamoeba dispar, but not *E. histolytica*, detected in a colony of chimpanzees in Japan

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Abstract Chimpanzees (*Pan troglodytes*) residing in the Kumamoto Primate Research Park, Sanwa Kagaku Kenkyusho, were surveyed for the presence of intestinal parasites. Stool samples from 107 chimpanzees were examined by microscopy after formalin-ether sedimentation. Of these animals, 100 were infected with at least 1 species of ameba. The positivity rates recorded were as follows: *Entamoeba coli*, 88%; *E. histolytica*/*E. dispar*, 48%; *E. hartmanni*, 15%; *Iodamoeba buetschlii*, 8%; *Endolimax nana*, 4%; and *Entamoeba chattoni*, 2%. Polymerase chain reaction (PCR) analysis to distinguish between *E. histolytica* and *E. dispar* was performed on these samples. *E. dispar* DNA was detected in 60 of 107 samples (56%), including 9 that had been microscopically determined to be negative for *E. histolytica*/*E. dispar*. In contrast, no *E. histolytica* DNA was detected in the 107 samples. Zymodeme analysis indicated that 10 isolates were *E. dispar*. When 104 chimpanzees were examined serologically for *E. histolytica* infection, 1 sample was scored as positive by indirect hemagglutination and another was found to be positive by an indirect fluorescent antibody test. However, both specimens were borderline-positive and were clearly negative in other tests, suggesting that they might be false-positives. These results demonstrate that the pathogenic *E. histolytica* was absent in this colony, regardless of the high degree of prevalence of other amebas. For an accurate diagnosis, PCR analysis is recommended in addition to microscopic examination.

H. Tachibana (✉) · X.-J. Cheng
Department of Infectious Diseases,
Tokai University School of Medicine, Bohseidai, Isehara,
Kanagawa 259-1193, Japan
e-mail: htachiba@is.icc.u-tokai.ac.jp
Fax: +81-463-955450

S. Kobayashi
Department of Tropical Medicine and Parasitology,
School of Medicine, Keio University, 35 Shinanomachi,
Shinjuku-ku, Tokyo 160-8582, Japan

Y. Fujita · T. Udono
Kumamoto Primate Research Park,
Sanwa Kagaku Kenkyusho Co., Ltd.,
990 Nishikuroiwa, Ohtao, Misumi,
Kumamoto 869-3201, Japan

Introduction

Amebiasis, caused by infection with the protozoan *Entamoeba histolytica*, is one of the most important parasitic diseases of humans. It has been estimated that 50 million people develop hemorrhagic amebic colitis and extraintestinal abscesses, resulting in 100,000 deaths annually (Walsh 1988). In addition to its occurrence in humans, *E. histolytica* is known to be found in captive and wild-trapped nonhuman primates such as macaque monkeys, orangutans, and baboons (Myers and Kuntz 1968; Reardon and Rininger 1968; Sano et al. 1980; Eberhard 1981; Collet et al. 1986; Beaver et al. 1988; Nasher 1988; Ghandour et al. 1995; Muriuki et al. 1998). Therefore, *E. histolytica* infections in nonhuman primates may have zoonotic importance (Smith and Meerovitch 1985).

Recently, *E. histolytica* has been reclassified into two species, i.e., *E. histolytica* Schaudinn, 1903 and *E. dispar* Brumpt, 1925, on the basis of biochemical, immunological, and genetic differences (Diamond and Clark 1993). The two species are morphologically indistinguishable, but only *E. histolytica* causes invasive amebiasis. Accordingly, it is necessary that the worldwide prevalence of *E. histolytica* infection be reevaluated after its distinction from *E. dispar* (WHO 1997).

The chimpanzee, *Pan troglodytes*, is the primate species taxonomically closest to humans. However, little is known about the prevalence of *E. histolytica* and *E. dispar* in wild and captive chimpanzees (Sargeaunt et al. 1982). The Kumamoto Primate Research Park in Japan has the biggest colony of chimpanzees in the Orient. The chimpanzees, originally imported from West Africa and used in medical experiments, were thereafter reared and bred in the Primate Research Park.

Previously, Kagei et al. (1988) had demonstrated a high incidence of intestinal parasitic infections, including *E. histolytica*/*E. dispar* infections, in this colony by stool examination.

The present study was undertaken for the examination of amebic infections in the colony, with special interest being directed at differentiation between *E. histolytica* and *E. dispar*. Polymerase chain reaction (PCR) and zymodeme analyses were used to distinguish between the two species. Serological examinations were also performed to survey the extent of *E. histolytica* infection.

Materials and methods

Chimpanzees

The Kumamoto Primate Research Park, Sanwa Kagaku Kenkyusho Co., Ltd., is located on the side of a hill in Misumi Town, Kumamoto Prefecture, overlooking the Ariake Sea. The area is rich in greenery and mild in climate. A total of 108 chimpanzees were in the Primate Research Park when the survey was carried out in December 1996. Of these, 42 chimpanzees (18 males and 24 females aged 15–26 years) had been imported from West Africa and used for hepatitis research. They were sent to the Park in 1982. The remaining 66 chimpanzees (32 males and 34 females aged 0–13 years) were bred in the colony. The chimpanzees were reared individually, in pairs, or in groups in cages of ample size.

Stool examination

Stool samples were collected from all of the chimpanzees in the Park except for one female infant in an incubator. These samples were examined microscopically following formalin-ether sedimentation.

PCR analysis

One-half of every formalin/ether-sedimented sample was washed twice with phosphate-buffered saline and then used for extraction of DNA as previously described (Rivera et al. 1996). An aliquot of isolated DNA was subjected to PCR amplification using two sets of primers, p11 (5′-GGAGGAGTAGGAAAGTTGAC-3′) and p12 (5′-TTCTTGCAATTCCTGCTTCGA-3′) for *Entamoeba histolytica* and p13 (5′-AGGAGGAGTAGGAAAATTAGG-3′) and p14 (5′-TTCTTGAAACTCCTGTTTCTAC-3′) for *E. dispar* (Tachibana et al. 1991a). A total of 30 cycles of PCR were performed as follows: denaturation at 94 °C for 60 s, annealing at 59 °C for 90 s, and polymerization at 72 °C for 90 s. An initial denaturation step of 4 min at 94 °C and a final polymerization step of 7 min at 72 °C were also included.

Zymodeme analysis

Fresh stool samples were inoculated into Robinson's medium (Robinson 1968). Zymodeme analysis of trophozoites was performed according to the procedure described by Sargeaunt (1988).

Serology

An indirect hemagglutination test (IHA) was performed using a commercial kit (Japan Lyophilization Laboratory, Tokyo, Japan). Titers of 1:80 were judged to be borderline-positive according to the manufacturer's instructions. An indirect fluorescent antibody (IFA) test was also performed as previously described, using formalin-fixed trophozoites of *E. histolytica* HK-9 as the antigen (Tachibana et al. 1990). Fluorescein isothiocyanate-conjugated goat antibody to human IgG (Medical and Biological Laboratories, Nagoya, Japan) was used as the secondary antibody. Titers of 1:64 were scored as borderline-positive in the test.

Results

Stool examination

Microscopic examination of formalin/ether-sedimented stools revealed high rates of amebic infection in both groups of chimpanzees, i.e., those born in Japan, which involved infants, juveniles, and adolescents, and those imported from West Africa, which were adults (Table 1). Amebic cysts were detected in 100 of 107 chimpanzees examined. The most prevalent ameba in the colony was *Entamoeba coli*, with an incidence of 88%. *E. histolytica*/*E. dispar* (48%), *E. hartmanni* (15%), *Iodamoeba buetschlii* (8%), *Endolimax nana* (4%), and *E. chattoni* (2%) followed in descending order. Two other protozoan parasites, *Chilomastix mesnili* (7%) and *Giardia intestinalis* (6%), were also detected. In addition, eggs of two nematodes, *Trichuris trichiura*

Table 1 Detection of intestinal parasites by formalin-ether sedimentation in 107 imported or Japan-bred chimpanzees

Species	Bred in Japan (Age 0–13 years) $n = 65$	Imported (Age 15–26 years) $n = 42$	Total $n = 107$
Amebas:			
Entamoeba coli	55 (85%)	39 (93%)	94 (88%)
E. histolytica/*E. dispar*	28 (43)	23 (55)	51 (48)
E. hartmanni	7 (11)	9 (21)	16 (15)
E. chattoni	2 (3)	0 (0)	2 (2)
Endolimax nana	2 (3)	2 (5)	4 (4)
Iodamoeba buetschlii	2 (3)	7 (17)	9 (8)
Flagellates:			
Chilomastix mesnili	3 (5)	4 (10)	7 (7)
Giardia intestinalis	5 (8)	1 (2)	6 (6)
Nematodes:			
Trichuris trichiura	14 (22)	4 (10)	18 (17)
Strongyloides fulleborni	4 (6)	0 (0)	4 (4)

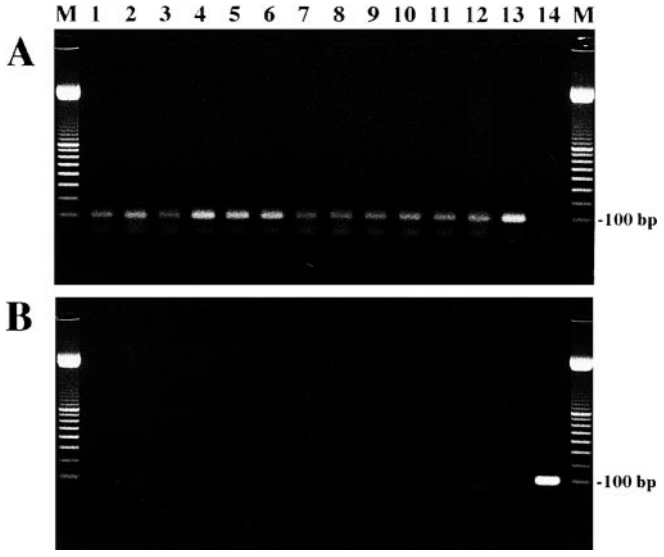

Fig. 1A, B Agarose gel separation of PCR products amplified by two pairs of primers, **A** p13 and p14 for *Entamoeba dispar* and **B** p11 and p12 for *E. histolytica*. Template DNA was extracted from formalin/ether-sedimented stool samples (*Lanes 1–12 E. histolytica*/*E. dispar*-positive samples from chimpanzees, *lane 13 E. dispar* control, *lane 14 E. histolytica* control, *M* DNA size marker – 100-bp ladder)

(17%) and *Strongyloides fulleborni* (4%), were found. The incidence of *G. intestinalis*, *T. trichiura*, and *S. fulleborni* was lower in adult chimpanzees than in younger animals.

PCR analysis

PCR amplification of DNA extracted from formalin/ether-sedimented fractions of stools using primers p13 and p14 yielded 101-bp products in 60 of 107 samples, indicating the existence of *E. dispar* DNA (Fig. 1A, Table 2). These PCR-positives included all 51 of the samples that were microscopically positive for *E. histolytica*/*E. dispar*. In addition, 9 of the *E. histolytica*/*E. dispar*-negatives yielded PCR products. Microscopically, all 9 were positive for *E. coli* and 2 were positive for *E. hartmanni*. On the other hand, when primers p11 and p12 were used, no product was obtained, which demonstrated that *E. histolytica* was not present (Fig. 1B).

Zymodeme analysis

Only 11 isolates were successfully cultured in Robinson's medium. Such a low return might be attributable to the accidental exposure of the cultures to an inappropriate temperature overnight. For 10 of the 11 isolates the zymodemes were as follows: Z-I, 4 isolates; Z-III, 4 isolates; and Z-IV, 2 isolates. This finding indicates that all 10 of these isolates were *E. dispar*. The remaining zymodeme showed an *E. coli* pattern.

Serology

In a search for invasive amebiasis the serum titers of antibody to *E. histolytica* were obtained (Table 3). Of 104 serum samples, 1 with a titer of 1:80 was scored as positive by IHA. Another sample with a titer of 1:64 was found to be positive by IFA.

Discussion

Several coprology surveys have demonstrated *Entamoeba histolytica*/*E. dispar* infection in chimpanzees as well as other nonhuman primates (Reardon and Rininger 1968; Sargeaunt et al. 1982; Kagei et al. 1988). One isolate, obtained in a zoo, was analyzed and identified as *E. dispar* by its zymodeme pattern (Sargeaunt et al. 1982). However, chimpanzee fatalities, apparently due to amebiasis, have been reported (Fremming et al. 1955; Miller and Bray 1966).

The present study demonstrated genetically that all of the *E. histolytica*/*E. dispar* amebas detected by stool examination were *E. dispar* and that no coinfection with *E. histolytica* existed. The possibility that *E. dispar* might have been introduced into the colony had to be slight because the chimpanzees in the colony had been reared in well-conditioned, modern facilities. Therefore, it is likely that *E. dispar* exists in West Africa, although we cannot exclude the possibility that *E. histolytica* might have been eliminated during rearing. To our knowledge, this is the first report concerning the prevalence of *E. histolytica* and *E. dispar* infections in a relatively large number of chimpanzees.

The parasites monitored in the present study had previously been studied by Kagei et al. (1988). Although the incidence of *E. coli* increased markedly from 28% to

Table 2 Detection of *E. histolytica* and *E. dispar* DNA in stool samples by PCR analysis and its comparison with microscopy

Microscopy	Number of samples	PCR			
		E. dispar	*E. histolytica*	*E. histolytica* and *E. dispar*	Negative
E. histolytica/*E. dispar*	51	51	0	0	0
Other amebas	49	9	0	0	40
Negative	7	0	0	0	7
Total	107	60	0	0	47

Table 3 Serological findings recorded for *E. histolytica* in 104 chimpanzees

Indirect hemagglutination test	Indirect fluorescent antibody test	
	Negative	Positive
Negative	102	1
Positive	1	0

88%, that of *E. histolytica*/*E. dispar*, 51–48%, remained essentially unchanged. Such a disparity may be attributable to differences between the two species with regard to the infectivity of cysts and/or the density of cysts in stools. A major route in the transmission of amebas may involve passage from the mother to the infant during rearing (Sakakibara et al. 1982). Support for this possibility would be provided by the observation that of the 12 infant chimpanzees in the colony that were less than 2 years of age, 9 were infected with *E. coli* and 3, with *E. histolytica*/*E. dispar* (data not shown). The low incidence of nematode infection in adult chimpanzees was in accord with previous findings (Kagei et al. 1988).

In the present study, two sera were scored as positive by IHA or IFA. It is well known that *E. dispar* shares common antigens with *E. histolytica* (Tachibana et al. 1991b; Sharma et al. 1994). Indeed, since the two chimpanzees were infected with *E. dispar*, it is probable that the observed antibodies were elicited by *E. dispar*. In any event, the titers that were only borderline-positive in 1 test and clearly negative in the other suggest that these 2 scores might be false-positives.

PCR analysis detected *E. dispar* DNA in 9 *E. histolytica*/*E. dispar*-negative samples. Since all 9 samples were *E. coli*-positive and 2 were *E. hartmanni*-positive, a possible explanation might be that in mixed populations, *E. histolytica*/*E. dispar* could be overlooked during microscopy. The PCR system using two pairs of primers for both *E. histolytica* and *E. dispar* appears to be sufficiently specific and sensitive, even for formalin-fixed stool samples containing cysts or for cultured samples containing trophozoites (Tachibana et al. 1991a; Rivera et al. 1996, 1998).

E. histolytica infection in nonhuman primates is a problem not only for the animals but also for humans, as the former serve as a zoonotic source. Indeed, transmission of amebas from nonhuman primates to humans has been reported elsewhere (Sargeaunt et al. 1982). Therefore, for an accurate diagnosis the PCR-based analysis of amebas is recommended in addition to microscopic examination.

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