2003, 217 in 2004, 282 in 2005, and 277 in 2006) (Table 1). All individuals provided informed consent. Outpatient age was the only additional information. The anti-EH antibody-positive sera were examined for anti-*Chlamydia trachomatis* (CT) and anti-TP antibodies as indicators of STDs.

ELISA: EH antigens were prepared from axenically cultured EH (HM-1: ATCC No. 50527). The antigen was diluted with 0.05 M bicarbonate buffer to yield a concentration of 5 μg/mL. The diluted antigen (100 μL) was pipetted into each well of the microplate (Nunc-Immuno Module; Nunc Co., Roskilde, Denmark; Cat. No. 469078) and sensitized by incubation for 2 h at 37°C (7). After washing with a buffer (0.15 M phosphate buffer (PB) containing 0.05% Tween 20, pH 7.2; PB/T), 100 μL of the serum samples diluted 1:200 with a dilution buffer (PB/T containing 1% bovine serum albumin, BSA) were pipetted into the microwells followed by incubation for 40 min at 37 °C. The microplate was washed 3 times with PB/T after incubation, and 100 μL of 1:8000 diluted peroxidase-conjugated anti-human IgG rabbit serum (ICN-Cappel Inc., Aurora, OH, USA; Cat. No. 55221) was added, followed by incubation for 40 min at 37°C. After washing with PB/T, the substrate solution comprising 0.03% 2,2'-azino-bis(3-ethylbenzo thiazoline-6-sulfonic acid) diammonium salt (ABTS; Sigma Co., Cat. No. A1888), 0.01% H_2O_2 in 10 mL of 0.1 M Na_2HPO_4, and 10 mL of 0.1 M citric acid was added to each well. After 7 min, 50 μL of 1.25% NaF solution was added to arrest color development, and an ELISA reader (MTP-120) (Corona Electric Co. Ltd., Ibaragi, Japan) was used to measure the absorbance at 405 nm. The cut-off S/N optical density (OD) value, calculated using the average OD of negative sera from 5 healthy individuals, was set at 3.

Serological test for CT and TP infections: Anti-CT IgG and IgA antibodies were measured using a solid-phase enzyme immunoassay kit (Peptide-Chlamydia IgG and

IgA; Ani Labsystems Ltd., Oy, Vantaa, Finland).

Nontreponemal anti cardiolipin (CL) antibodies were detected using 2 kits (biologic false-positive tests for syphilis), i.e., slide test antigen (DS Pharma-Biomedicals, Osaka, Japan) and rapid plasma reagin (RPR) test (Sanko Junyaku, Tokyo, Japan). The sera that tested positive by these kits were retested by using the TP passive hemagglutination (TPHA) kit (Fujirebio Inc., Tokyo, Japan) for detection of anti-TP antibody. The OD values of the positive and negative control sera for quality control and the OD value of the positive control of the kits were measured during each test run of all the serological tests.

ELISA with the antigens from the virulence-augmented amoebae: Based on the hypothesis that the amount of antigenic substances also decreased simultaneously with loss of virulence, we attempted to design an ELISA with better sensitivity in the following manner: 1) HM-1 and LA526 strains cultured axenically for 3 days in the TYI-S-33 medium were inoculated (dose, 1×10^6 amoebae/0.1 mL/head) into the left hepatic lobes of female Syrian golden hamsters (age, 3–4 weeks) (8). 2) On 6th day of inoculation, the hamsters were sacrificed and the livers dissected aseptically, the amoebic abscesses isolated from each of the livers were minced fine and crushed using scissors for medical use in the TYI-S-33 medium (9). 3) After removing the tissue debris from the amoebic cell suspensions and washing twice in the TYI-S-33 medium by centrifugation ($175 \times g$ for 3 min), the 2 EH strains were cultured axenically in the TYI-S-33 medium. They were named LHM-1 and LLA526. The long term axenically cultured HM-1 was passaged 16 times through hamster liver due to significant reduction in the virulence, whereas the LA526 was passaged only once because it was newly isolated from the pus of a human amoebic liver abscess only 8 months ago. 4) LHM-1 and LLA526 were mass cultured within 2 weeks after transferring into

TYI-S-33 medium from the amoebic liver abscesses and they were harvested and washed twice in phosphate buffered saline (PBS) by centrifugation (175 × *g* for 3 min), and suspended in 5 mL of distilled water, followed by intermittent sonication (UH-150; SMT Co. Ltd, Tokyo, Japan) at 10 kHz for 5 min in an ice bath. 5) The sonicated suspensions were then centrifuged at 9,100 × *g* for 30 min, and the protein concentrations of the aqueous soluble extracts were measured by Bradford's method (10). 6) LHM-1 and LLA526 antigens were sensitized at a concentration of 0.5 μg/well according to the procedures described above.

Each serum sample was tested in triplicate for each of the 3 antigens, i.e., LHM-1, LLA526, and HM-1, and the average OD values were calculated. The sensitivity of ELISA for each of the 3 antigens was compared with the positive serum samples of 5 patients clinically diagnosed to have amoebic liver abscesses and 5 mentally handicapped persons in a rehabilitation institution for the intellectually impaired in Japan who were almost free from symptoms of amoebiasis but were positive for EH cysts on microscopy and for EH antigen when tested by using an EH-specific antigen detection kit (*E. histolytica* II kit; TechLab, Blacksburg, Va, USA). In each of the 10 human serum samples obtained as described above from the cases of amoebic liver abscess and asymptomatic cyst passers, the ratio between the S/N values (OD value of serum sample (S)/average OD of negative sera from 5 healthy individuals (N)) of ELISA with the LHM-1 and HM-1 antigens and that between the S/N values of ELISA with the LLA526 and HM-1 antigens was determined.

RESULTS

Seroprevalence of anti-EH antibodies in the female population: During the 4 years, i.e., from 2003 to 2006, in the 981 sera obtained from the study population, the

seroprevalence of anti-EH (HM-1) antibodies increased every year. In 2005 and 2006, the annual positive rate was >5%; the average of the annual positive rates over the 4 years was 4.1% (40/981) (Table 2). In addition, 60%, i.e., 24/40 of these cases were also positive for anti-CT antibodies—an indicator of STDs. On the contrary, none of the cases were positive for anti-CL antibodies (retest by TPHA kit was not performed). The strong positive correlation between seropositivity for anti-EH and anti-CT antibodies suggested sexual transmission of EH in the female population. In terms of age, the highest number of individuals positive for anti-EH antibodies was observed in 11 individuals aged 25–29 years and in 9 individuals aged 30–34 years. The number of individuals positive for anti-CT antibodies correlated with the number of those positive for anti-EH antibodies (Table 3).

Comparison of S/N values of ELISA with 3 different antigens: The results of a pilot study in which the ELISA with LHM-1 and LLA526 antigens was conducted using sera from 5 human cases each of amoebic liver abscess and asymptomatic cyst passers are shown in Figure 1. The average ratio (1.324) between S/N values of ELISA with the LHM-1 and HM-1 antigens and that (1.254) between S/N values of ELISA with the LLA526 and HM-1 antigens increased significantly in only asymptomatic cases with low S/N values ($p < 0.05$ by *t*-test) and not in ALA cases (1.048 and 1.006, respectively; $p > 0.05$ by *t*-test).

The 40 anti-HM-1 antibody-positive sera as detected by ELISA were classified into 2 groups based on the magnitude of the S/N values (i.e., groups I and II with S/N values ≥10 and <10, respectively). The tendency of ELISA with LHM-1 and LLA526 antigens to yield significantly higher S/N values ($p < 0.01$ by *t*-test) was also confirmed in seropositive cases from among the present study population with low S/N values (<10) by ELISA using the HM-1 antigen (Figure 2).

DISCUSSION

In Japan, the MSM population is still thought to be a major high-risk group for STDs. However, our study provided evidence indicating that the seroprevalence of the EH infection in the female population of Tokyo is increasing annually.

In addition, the result that 60% of the female study population who were anti-EH antibody-positive were also positive for anti-CT antibodies, an indicator of STD, along with the diversity of sexual behavior suggested that a major proportion of females positive for anti-EH antibodies were possibly infected with EH by sexual transmission. We do not fully understand why none of the cases were positive for anti-CL antibodies in the female population unlike the MSM population (11, 12). We are, currently, conducting further epidemiological studies on the route of EH infection in the female population.

The tendency of ELISA using the LHM-1 and LLA526 antigens to yield statistically higher S/N values ($p < 0.01$ by *t*-test) was evident only in the positive cases with low S/N values (<10) among the present female study population. The active antigenic substance that brought about this effect could not be identified in the present study. Despite the necessity for further evaluation, the improved ELISA is expected to be an effective method for detecting anti-EH antibodies from such asymptomatic patients who have low antibody titers, and the hamster liver-passaged EH may be applied as a sensitive antigen to other serodiagnostic methods, such as dot-ELISA (13) and immunofluorescence antibody (IFA) test (14).

The control of amoebiasis should start with efforts toward raising the awareness of the general public regarding the risk of infection by sexual transmission because of their indifference to STD, and establishing more sensitive and simple mass

examination methods, such as the newly designed ELISA using the antigen extracted from the virulence-augmented EH strains, that have a better sensitivity for the diagnosis of amoebiasis.

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REFERENCES

1) Markell, E. K., Havens, R. F, Kuritsubo, R. A., et al. (1984): Intestinal protozoa in homosexual men of the San Francisco Bay area: prevalence and correlates of infection. Am. J. Trop. Med. Hyg., 33(2), 239–245.

2) Schmerin, M. J., Gelston, A. and Jones, T. C. (1977): Amebiasis. An increasing problem among homosexuals in New York City. JAMA, 238(13), 1386–1387.

3) Takeuchi, T., Okuzawa, E., Nozaki, T., et al. (1989): High seropositivity of Japanese homosexual men for amebic infection. J. Infect. Dis., 159, 808.

4) Nozaki, T., Motta, S. R., Takeuchi, T., et al. (1989): Pathogenic zymodemes of *Entamoeba histolytica* in Japanese male homosexual population. Trans. R. Soc. Trop. Med. Hyg., 83(4), 525.

5) Infectious Disease Surveillance Center (2007): Amebiasis in Japan. IASR, 28, 103–104, http://idsc.nih.go.jp/iasr/iasr-ge1.html

6) Infectious Disease Surveillance Center (2005): The Annual Data Summary consists of the spreadsheet files, which includes the number of reported cases for Category I, II, III, IV and V diseases from 1999 through 2005, http://idsc.nih.go.jp/idwr/ydata/index-e.html

7) Kanwar, J. R. and Vinayak, V. K. (1991): A comparative efficacy of plate ELISA and dot ELISA to detect antiamoebic antibodies in clinical patients. Trop. Geogr. Med., 43(3), 261–265.

8) Diamond, L. S., Phillips, B. P. and Bartgis, I. L. (1974): A comparison of the virulence of nine strains of axenically cultivated *E. Histolytica* in hamster liver. Arch. Invest. Med. 5:423-426.

9) Diamond, L. S., Harlow, D. R. and Cunnick, C. C. (1978): A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. Trans. R. Soc. Trop. Med. Hyg., 72, 431–432.

10) Bradford, M. M. (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72, 248–254.

11) Takeuchi, T., Kobayashi, S., Asami, K., et al. (1987): Correlation of positive syphilis serology with invasive amebiasis in Japan. Am. J. Trop. Med. Hyg., 36, 321–324.

12) Okusawa, E., Kobayashi, S., Miyahira, Y., et al. (1991): Study on clinical forms and demographic backgrounds of Japanese adult males with invasive amebiasis according to the history of sexual practice. Jpn. Arch. Sex. Transm. Dis., 1, 153–156 (in Japanese).

13) Yamaura, H., Araki, K., Kiuchi, K., et al. (2003): Evaluation of dot-ELISA for serological diagnosis of amebiasis. J. Infect. Chemother., 9, 25–29.

14) Ambroise-Thomas, P. and Truong, T. K. (1972): Fluorescent Antibody Test in Amebiasis. Am. J. Trop. Med. Hyg., 21(6), 907–912.

Figure legends

Figure 1. Ratio of the S/N values of ELISA with better sensitivity performed using the antigens from hamster liver-passaged LHM-1 and LLA526 strains to the S/N values of ELISA using the antigen from the HM-1:IMSScl6 (HM-1) strain in the clinical serum samples of amoebiasis (S/N values: OD of serum sample (S)/average OD of negative sera (N)].

The mean of the ratios of triplicate ELISA are plotted. I: Samples (n = 5); anti-HM-1 antibody-positive sera from the clinical patients of amoebic liver abscess. The S/N values of ELISA with LHM-1 and LLA526 antigens did not increase significantly ($p > 0.05$ by the *t*-test). II: Samples (n = 5); anti-HM-1 antibody-positive sera from the mentally handicapped individuals admitted to a rehabilitation institution for the intellectually impaired in Japan. The S/N values of ELISA with LHM-1 and LLA526 antigens increased significantly ($p < 0.05$ by the *t*-test).

Figure 2. Ratio of S/N values of ELISA performed using antigens from the LHM-1 and LLA526 strains to the S/N values of conventional ELISA performed using the antigen from the HM-1 strain in female outpatients from a gynecologist's office. The mean of the ratios of triplicate ELISA are plotted.

I: Samples (n = 16); anti-HM-1 antibody-positive sera with high S/N values (≥10). The S/N values of ELISA with LHM-1 and LLA526 antigens did not increase significantly ($p > 0.05$ by the *t*-test). II: Samples (n = 24); anti-HM-1 antibody-positive sera with low S/N values (<10). The S/N values by ELISA with LHM-1 and LLA526 antigens increased significantly ($p < 0.01$ by *t*-test).

Table 1. Study samples in age categories from 2003 to 2006

Age	2003	2004	2005	2006	Total
< 20	15	18	22	12	67
20–24	53	56	71	79	259
25–29	69	59	83	90	301
30–34	41	54	57	41	193
35–39	10	18	25	24	77
40–44	8	4	8	12	32
45–49	2	1	8	1	12
50 <	1	2	5	7	15
unknown	6	5	3	11	25
	205	217	282	277	981

Table 2. Seroprevalence of anti-*Entamoeba histolytica* antibodies in the female outpatients from a gynecologist's office, Tokyo, Japan, by enzyme-linked immunosorbent assay (ELISA) from 2003 to 2006

Year	No. of samples	No. of positives	Positive rate %	No. of positives for anti-CT antibodies[1)]
2003	205	3	1.5	2
2004	217	8	3.7	6
2005	282	14	5.0	7
2006	277	15	5.4	9
Total	981	40	4.1	24

1): Number of positives for anti-*Chlamyidia trachomatis* (CT) antibodies that were also positive for anti-*Entamoeba histolytica* antibodies.

Table 3. Age distribution of the female outpatients from a gynecologist's office with positive for anti-*E. histolytica* antibodies

Year	20–24	25–29	30–34	35–39	40–44	45–49	50 <
2003	1	1 (1)		1 (1)			
2004		2 (1)	3 (3)	2 (2)			
2005	1	2 (1)	3 (1)		3	4 (3)	2 (2)
2006	3 (2)	6 (5)	3 (2)		2		1
Total	5 (2)	11 (8)	9 (6)	3 (3)	5	4 (3)	3 (2)

Number of positives for anti-*Chlamyidia trachomatis* (CT) antibodies that were also positive for anti-*Entamoeba histolytica* antibodies are provided in parentheses

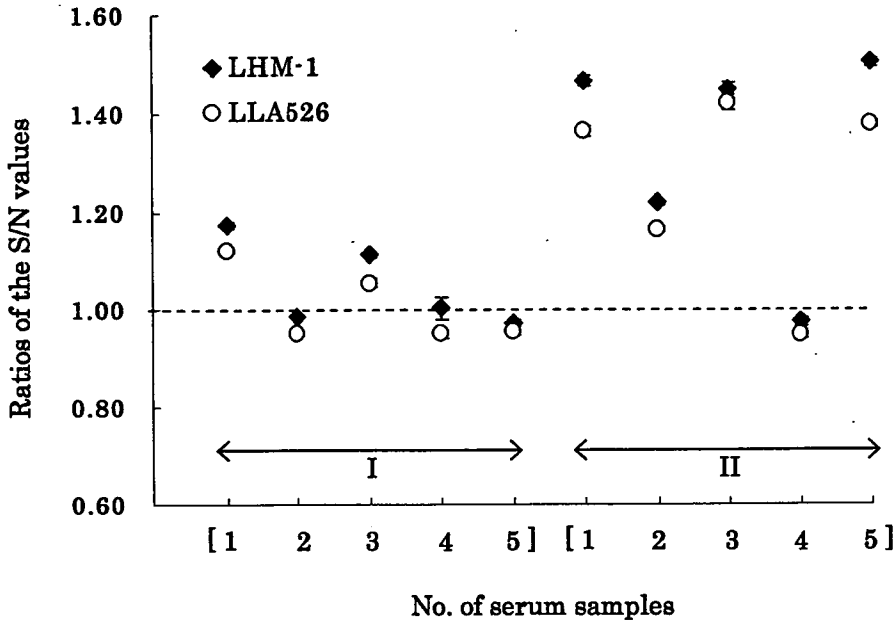

Figure 1

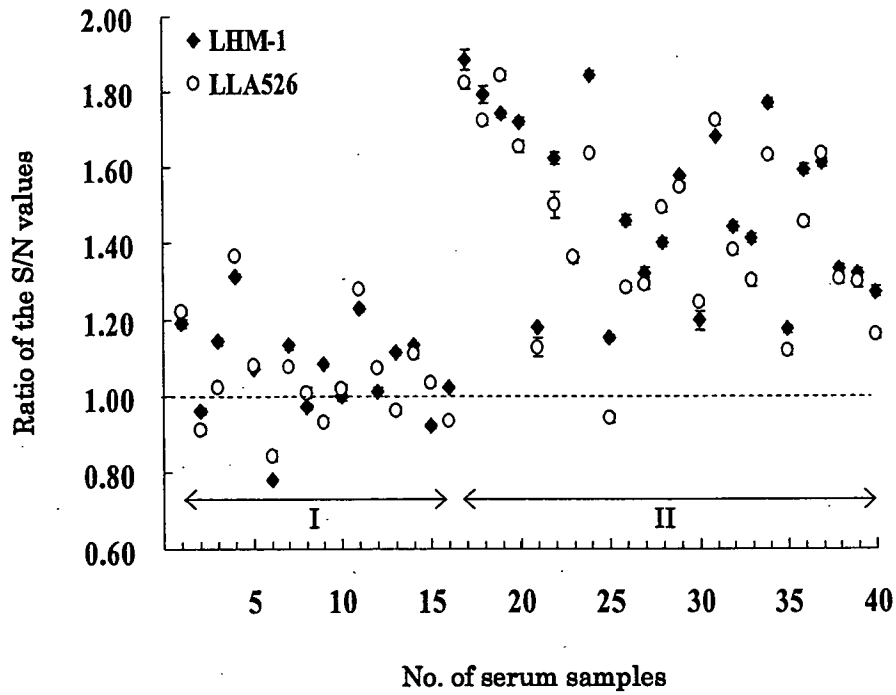

Figure 2

A SURVEY OF AMOEBIC INFECTIONS AND DIFFERENTIATION OF AN ENTAMOEBA HISTOLYTICA-LIKE VARIANT (JSK2004) IN NONHUMAN PRIMATES BY A MULTIPLEX POLYMERASE CHAIN REACTION

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Abstract: We previously reported a pathogenic Entamoeba histolytica-like variant (JSK2004 strain) with genetic variations and a novel isoenzyme pattern isolated from a De Brazza's guenon in a Tokyo zoo in Japan. In this study, we further designed and established a multiplex polymerase chain reaction (PCR) assay that could distinguish the JSK2004 type E. histolytica-like variant (JSK04-Eh-V) from E. histolytica and E. dispar using 3 newly designed primer sets for amplifying each specific DNA fragment from their small-subunit rRNA genes. Forty-seven primates (11 species) from the zoo were surveyed by multiplex PCR to assess the prevalence of JSK04-Eh-V infection, which was recognized in 6 individuals (4 species): Abyssinian colobus monkey, De Brazza's guenon including the individual from whom JSK2004 was isolated, white-faced saki, Geoffroy's spider monkey, and the autopsied individuals of Abyssinian colobus and Geoffroy's spider monkey that died of amoebic liver abscess. Their DNA samples were also analyzed for specific genotypes based on the nucleotide sequencing of 2 protein-coding [chitinase and serine-rich E. histolytica protein] genes and the protein-noncoding locus 1-2 that was used for fingerprinting of the E. histolytica strain. These studies suggested that the E. histolytica-like variant infection in this zoo was caused by the same type, i.e., JSK04-Eh-V. We designed an axenic culture medium (yeast extract-iron-maltose-dihydroxyacetone-serum; YIMDHA-S) based on the yeast extract-iron-gluconic acid-dihydroxyacetone-serum (YIGADHA-S) medium which is designed for axenic culture of E. dispar; our new medium could be used for axenically culturing E. histolytica, JSK04-Eh-V, and E. dispar in a single medium.

Keywords: Entamoeba histolytica-like variant, JSK2004 strain, multiplex PCR, nonhuman primates, YIMDHA-S medium, zoo.

INTRODUCTION

Amoebiasis is a zoonotic protozoal infectious disease caused by Entamoeba histolytica. The estimated incidence of amoebiasis in humans is approximately 50 million per year, and it has caused nearly 70,000 human deaths.[18] In 1997, E. histolytica was reclassified into 2 species: E. histolytica and Entamoeba dispar (nonpathogenic); earlier, this differentiation was difficult because of morphogenetic and phylogenetic similarities.[1,19] This classification is based on the differences in the isoenzyme patterns (zymodemes), the detection of the E. histolytica-specific antigen, and E. histolytica- and E. dispar-specific DNA fragment amplification by PCR.[3,5]

In Japan, particularly during the last decade, E. histolytica infections have note been detected in nonhuman primates by PCR.[12,14] However, 3 recent articles reported 3 different pathogenic E. histolytica-like variants showing subtle variations in the small-subunit rRNA

(SSU rRNA) gene sequences isolated from cynomolgus monkey (Macaca fascicularis),[15] rhesus monkey (Macaca mulatta),[13] and De Brazza's guenon (Cercopithecus neglectus)[12]. The JSK2004 type E. histolytica variant (JSK04-Eh-V)[11] has an SSU rRNA gene homology of 99.10% with E. histolytica and 98.47% with E. dispar. Previously, we reported that the existing multiplex PCR[2] technique that targeted the specific region of the SSU rRNA gene sequence of E. histolytica did not yield the genomic DNA products of an axenic strain (JSK2004) of the E. histolytica-like variant from a De Brazza's guenon due to the variation in the nucleotide sequence of the gene.[11]

In the present study, a new multiplex PCR assay that is capable of distinguishing the JSK04-Eh-V from E. histolytica and E. dispar was designed, and the prevalence of JSK04-Eh-V infection in the primates of a zoo in Japan was surveyed using this assay. In addition, we investigated the identity of the JSK04-Eh-V strains in the zoo by analyzing their polymorphic genotypes as a fingerprint for identifying the strain of E. histolytica.

We also surveyed the incidence of other amoebic infections in primates and designed the first axenic culture medium that would support the growth of E. histolytica, JSK04-Eh-V, and E. dispar in a single medium.

MATERIALS AND METHODS

Primates

In order to assess the prevalence of infection with JSK04-Eh-V, we surveyed 47 captive individuals of 11 primate species from the Tokyo zoo in Japan where JSK2004 (JSK04-Eh-V) had been isolated from a De Brazza's guenon.[11] The primates included (1) 3 De Brazza's guenons (Cercopithecus neglectus), (2) 11 Abyssinian colobus monkeys (Colobus guereza), (3) 2 ring-tailed lemurs (Lemur catta), (4) 2 mandrills (Mandrillus sphinx), (5) 1 lesser slow loris (Nycticebus pygmaeus), (6) 2 ruffed lemurs (Varecia variegata), (7) 1 northern night monkey (Aotus trivirgatus), (8) 7 Geoffroy's spider monkeys (Ateles geoffroyi), (9) 12 Japanese macaques (Macaca fuscata), (10) 5 white-faced sakis (Pithecia pithecia), and (11) 1 cotton-top tamarin (Saguinus oedipus). Each primate species was housed independently.

Microscopic examination and detection of the E. histolytica-specific antigen

Stool samples from each living individual were collected once daily for 3 days from 45 primates, that is, 3 samples per individual, for the following examinations. Prior to performing

multiplex PCR, all stool specimens were examined microscopically after concentrating the Entamoeba cysts by the formalin-ether sedimentation technique;[10] the specimens were also examined by using an E. histolytica-specific antigen detection kit (E. histolytica II kit; TechLab, Blacksburg, Virginia 24060, USA). Tissues samples obtained from the primates with liver abscess were paraffinized and stained with PAS and were examined for amoebae.

DNA preparation

The amoebic cysts in the stool specimens (from among the 3 collected stool specimens from each individual, the specimen in which the largest number of amoebic cysts were detected was provided for DNA preparation) were concentrated and partially purified using the modified formalin-ether sedimentation method, i.e., replacement of formalin with a phosphate-buffered solution (pH 7.4). Subsequently, the QIAamp® DNA stool mini kit (Qiagen GmbH, Hilden 40724, Germany; Catalogue no. 51504) was used to isolate the genomic DNAs of amoebae. The genomic DNAs of (1) the amoebae in the 2 pus samples obtained from the liver abscess of the autopsied Abyssinian colobus monkey and Geoffroy's spider monkey that died of amoebic liver abscess; (2) the 2 reference amoebic strains, HM-1:IMSS clone 6 (HM-1:IMSScl6 strain; E. histolytica) and SAW1734R clone AR (SAW1734RclAR strain; E. dispar), that were kindly supplied by Dr. Lois S. Diamond, NIH, USA; and (3) the JSK2004 clone 2 (JSK2004cl2; JSK04-Eh-V) were isolated by using the QIAamp DNA mini kit (Qiagen GmbH, Hilden 40724, Germany; Catalogue no. 51304).

Primers for multiplex PCR

The primers for multiplex PCR were designed based on the 2 SSU rRNA gene sequences of HM-1:IMSScl6 (E. histolytica; GenBank accession no. X65163) and SAW1734RclAR (E. dispar; GenBank accession no. AB282661) and the previously reported sequence of JSK2004cl2 (JSK04-Eh-V; GenBank accession no. AB426549). The 3 primer sets that were designed—EnthF/EnthR for E. histolytica, EntdF/EntdR for E. dispar, and EhvF/EhvR for JSK04-Eh-V—are listed in Table 1.

Primer specificity was tested by conducting multiplex PCR on 7 other intestinal parasitic protozoan and 1 nonprotozoan species: axenic trophozoites of E. moshkovskii (Laredo strain), E. invadens (IP-1 strain; ATCC no. 30994), and Giardia intestinalis (Portland-1 strain; ATCC no. 30888); cyst forms of E. coli and Cryptosporidium hominis; culture form of Blastocystis

hominis (nonprotozoan species) from human stool samples; and E. coli, E. chattoni, and E. hartmanni obtained from the stool samples of nonhuman primates.

Multiplex PCR

Amplification was performed in a reaction mixture (50 μL) containing 100 ng of the DNA samples, 25 μL of 2 × Multiplex PCR Master Mix (Qiagen GmbH, Hilden 40724, Germany; Catalogue no. 206143), and 2 μL of each primer at 10 mM. The touchdown method was used for thermal cycling. The cycling conditions were as follows: 15 min at 95°C followed by 40 cycles of denaturation at 94°C for 30 s, annealing for 40 s beginning at 61°C and ending at 56°C, and extension at 72°C for 1 min. The annealing temperature was lowered by 1°C after every 4 cycles until it reached 56°C, after which the same temperature was maintained till the end of the cycling process.

Semi-nested PCR for SSU rRNA

In cases where a minimal PCR product from the DNA of JSK04-Eh-V was obtained, a semi-nested PCR using the primer set EhvF/EhvR2 was performed (Table 1). For this second PCR (semi-nested PCR), amplification was performed in a reaction mixture (50 μL) containing 1 μL of the first PCR product, 1.0 U of exTaqDNA polymerase (Takara Bio Inc., Seta, Shiga 520-2134, Japan; Catalogue no. RR001A), 0.4 μM of each primer, and 0.25 mM of deoxynucleoside triphosphate. The following cycling parameters were used: (1) Taq activation at 94°C for 3 min; (2) 35 cycles of denaturation at 94°C for 40 s, annealing at 58°C for 40 s, and extension at 72°C for 1 min; and (3) extension at 72°C for 5 min.

PCR for E. chattoni and E. hartmanni

E. chattoni and E. hartmanni were identified by PCR assays using 2 primer sets, i.e., EchatF/EchatR[16] and EhartF/EhartR, respectively (Table 1). For E. hartmanni, a newly designed primer set based on its SSU rRNA sequence (GenBank accession no. AF149907) was used. These amplifications were performed in a reaction mixture (50 μL) containing 100 ng of the DNA sample, 1.0 U of LATaqDNA polymerase, 0.4 μM of each primer, and 0.25 mM of deoxynucleoside triphosphate. The following cycling parameters were used: (1) Taq activation at 94°C for 3 min; (2) 35 cycles of denaturation at 94°C for 40 s, annealing at 55°C

for 40 s, and extension at 72°C for 1 min; and (3) extension at 72°C for 5 min.

Polymorphic gene analysis

Genotyping of JSK04-Eh-V was re-examined to determine whether they have the same genotype as JSK2004cl2; this was performed based on the nucleotide sequences of 2 protein-coding (chitinase and SREHP) genes and the protein-noncoding locus 1-2[4,7,20] that was used as a fingerprint for identifying the E. histolytica strain. The primers used are shown in Table 1. These amplifications were performed in a reaction mixture (50 μL) containing 100 ng of the DNA sample, 1.0 U of LATaqDNA polymerase (Takara Bio Inc., Seta, Shiga 520-2134, Japan; Catalogue no. RR02AG), 0.4 μM of each primer, and 0.25 mM of deoxynucleoside triphosphate. The following cycling parameters were used: (1) Taq activation at 94°C for 3 min; (2) 35 cycles of denaturation at 94°C for 40 s, annealing at 50°C (chitinase, SREHP, and locus 1-2) or 56°C (SSU rRNA) for 40 s, and extension at 72°C for 1 min; and (3) extension at 72°C for 5 min.

Sequence analysis

The multiplex PCR products of SSU rRNA; the PCR products of chitinase, SREHP, and the locus 1-2 genes from the JSK04-Eh-V isolates; and the PCR products of SSU rRNA from E. chattoni and E. hartmanni were sequenced by using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California 94404, USA; Catalogue no. 4337455) and an ABI PRISM 3100 Genetic Analyzer.

YIMDHA-S medium

We designed an axenic culture medium, namely, yeast extract-iron-maltose-dihydroxyacetone-serum (YIMDHA-S), based on the yeast extract-iron-gluconic acid-dihydroxyacetone-serum medium (YIGADHA-S)[6] designed for the axenic culture of E. dispar, for the isolation and culture of E. histolytica, the E. histolytica-like variant, and E. dispar in a single medium. YIGADHA-S differs from YIMDHA-S in that gluconic acid—0.5% in the former—is replaced with an equal concentration of maltose in the latter. Another significant issue related to this culture system is that the growth of amoebae in YIMDHA-S is largely affected by the quality of the yeast extract. Accordingly, we tested the

effectiveness of several commercially available yeast extracts purchased from different manufacturers as an ingredient of YIMDHA-S. Except our old standard stock obtained from BBL (Becton Dickinson Co, MD 21030, USA; Catalogue no. 4311929; Lot no. 1000I9DHJT), among all the yeast extracts tested, only that obtained from Merck (Merck KGaA, Damstadt 64271, Germany; Catalogue no. 1.03753; Lot no.VM510453 539) was effective for constant subculture of the axenic strains of E. histolytica (HM-1:IMSScl6), E. dispar [AS16 IR isolated from human samples and CYNO 09:TPC isolated from the cynomolgus monkey (Macaca fascicularis)],[6] and JSK04-Eh-V (JSK2004) from the De Brazza's guenons that were subjected to a trial of axenic cultivation.

RESULTS

Specificity of multiplex PCR

Each PCR product from the genomic DNA of each of the axenic strains of E. histolytica (HM-1:IMSScl6), JSK04-Eh-V (JSK2004cl2), and E. dispar (SAW1734clAR) analyzed by multiplex PCR was obtained independently; the lengths of the fragments were 475 bp, 848 bp, and 195 bp, respectively (Figure 1), which was confirmed by individual nucleotide sequencing. The findings of multiplex PCR were reproducible during the practical trials using amoebic DNAs isolated from the stool samples and liver abscesses of humans and nonhuman primates infected with E. histolytica, JSK04-Eh-V, and E. dispar (Figure 1). The specificity of multiplex PCR was examined by analyzing the templates of the DNA extracted from 7 other intestinal parasitic protozoan and 1 nonprotozoan species as described under materials and methods. None of the PCR products were observed on multiplex PCR examination of these parasites (data not shown).

Sensitivity of multiplex PCR and semi-nested PCR

The sensitivity of multiplex PCR was found to be at least 200 cysts/100 mg of the stool sample for JSK04-Eh-V and 100 cysts/100 mg of the stool samples for E. histolytica and E. dispar. In an attempt to assess the sensitivity of the technique for mixed infections, 400 cysts or trophozites of JSK04-Eh-V could be detected and differentiated in the presence of 100 cysts/100 mg of the stool samples for E. histolytica and E. dispar.

Semi-nested PCR using the primer set EhvF/EhvR2 detected 50 cysts/100 mg stool sample for JSK04-Eh-V. In one case of the white-faced saki in which a minimal PCR product

from the DNA of JSK04-Eh-V was obtained, semi-nested PCR yielded the 434-bp-long PCR product.

Prevalence of amoebic infections in the zoo

Since a pathogenic JSK04-Eh-V strain was isolated from De Brazza's guenons in the Tokyo zoo, the prevalence of JSK04-Eh-V infection was surveyed using multiplex PCR and was detected in 6 individuals of 4 primate species: Abyssinian colobus monkey, De Brazza's guenon including the individual from which JSK2004 was isolated, white-faced saki, Geoffroy's spider monkey, and the autopsied samples of Abyssinian colobus monkey and Geoffroy's spider monkey that died of amoebic liver abscess diagnosed histopathologically (PAS-positive cells morphologically consistent with amoebae were identified in the paraffin section). Among the primates of the zoo, the infection rates with E. histolytica, E. dispar, and JSK04-Eh-V were 0% (0/47), 17% (8/47), and 13% (6/47), respectively (Table 2). Mixed infection with E. dispar and JSK04-Eh-V was not detected by multiplex PCR. The prevalence of the other species of amoebae examined microscopically, i.e., E. coli and Endolimax nana, were 30% (14/47) and 15% (7/47), respectively. The prevalence of E. chattoni and E. hartmanni examined microscopically and by PCR was 17% (8/47) and 11% (5/47), respectively (Table 2); their fragments were confirmed by nucleotide sequencing and corresponded to the sequence dates of E. hartmanni (GenBank accession no. AF149907) and E. chattoni (GenBank accession no. AF149912). The amplified PCR products are shown in Figure 2.

Polymorphic genes in JSK04-Eh-V isolates

Genotyping based on the nucleotide sequencing of the chitinase, SREHP, and locus 1-2 genes was applied to the genotyping of JSK04-Eh-V. The DNA samples of JSK04-Eh-V from each of the 6 primates were subjected to PCR to detect the fragments of the chitinase, SREHP, and locus 1-2 genes. We were able to sequence the PCR products of the chitinase genes from the DNA samples of 2 primates and JSK2004cl2 and the PCR products of the locus 1-2 genes from the DNA samples of 4 primates and JSK2004cl2; however, the PCR products of the SREHP genes were obtained only from the DNA sample of JSK2004cl2. There was perfect homology between (1) the sequences of the PCR products of the chitinase genes obtained from 2 primates (1 Geoffroy's spider monkey and 1 white-faced saki) and the PCR products

of the locus 1-2 genes from 4 primates (2 Abyssinian colobus monkeys, 1 Geoffroy's spider monkey, and 1 white-faced saki) and (2) the sequences of the 2 genes of JSK2004cl2 (locus 1-2; GenBank accession no. AB426704, chitinase; GenBank accession no. AB426705).

However, the sequence data of the chitinase and SREHP genes of the other 2 types of E. histolytica-like variants (GenBank accession nos. AB282755 and AB197935) isolated from the cynomolgus and rhesus monkeys was different as compared to the sequence data of the chitinase gene and SREHP gene (GenBank accession no. AB426706) of JSK2004cl2 (Figures 3 and 4).

Growth kinetics of amoebae in YIMDHA-S

The growth kinetics of axenically grown E. histolytica (HM-1:IMSScl6), E. dispar (AS 16 IR and CYNO 09:TPC), and JSK04-Eh-V (JSK2004) in YIMDHA-S are shown in Figure 5. These established axenic strains adapted to the YIMDHA-S culture conditions within 3 subcultures; thereafter, they were inoculated into the YIMDHA-S from the classic TYI-S-33[2] (HM-1:IMSScl6) or YIGADHA-S (AS 16 IR and CYNO 09:TPC) media.

DISCUSSION

The multiplex PCR for E. histolytica, JSK04-Eh-V, and E. dispar permits species identification in a single reaction mixture and is, therefore, more cost effective and useful for prevention of contamination of DNA samples.

Surveillance of the prevalence of JSK04-Eh-V infection among the primates in the zoo was conducted using multiplex PCR for differential diagnosis of E. histolytica, JSK04-Eh-V, and E. dispar. Multiplex PCR was confirmed as a useful method for the detection and identification of E. histolytica, JSK04-Eh-V, and E. dispar in nonhuman primates and even in humans, e.g., the zookeepers who are in contact with the primates, because the specificity and reproducibility of this technique were adequate for efficient surveillance of JSK04-Eh-V in the present study.

Concerning the microscopic stool examination process in this survey, amoebic cysts or trophozoites were not always detected in every stool sample obtained from individuals infected with JSK04-Eh-V and E. dispar. These cysts or trophozoites could be detected only in one- to two-thirds of the stool samples, despite the collection of samples from each individual primate once a day for 3 days. The results indicated that performing a stool examination per day (at least 3 times) on alternate days is necessary.

The JSK04-Eh-V strain of E. histolytica was detected by using the E. histolytica II kit, an E. histolytica-specific antigen (adhesin) detection kit. It is reported that one of the factors determining the pathogenicity of E. histolytica is the cytolysis of host cells that begins with the adhesion of the amoebae to the mucosal target cells of the large intestine via galactose/N-acetyl D-galactosamine-inhibitable (Gal/GalNAc) lectin.[8,9] The detection of the E. histolytica-specific antigen from JSK04-Eh-V by using the E. histolytica II kit indicated that the Gal/GalNAc lectin structure in JSK04-Eh-V is identical to that in E. histolytica.

Although the nucleotide sequence of the polymorphic SREHP gene from 5 primates except JSK2004cl2 could not be amplified by PCR, the polymorphic chitinase and locus 1-2 gene sequences from 3 and 6 primates, respectively, were observed to be identical. The reasons for the inability of PCR to amplify the SREHP gene were thought to be due to the small amount of JSK04-Eh-V DNA in the stool and liver abscess samples that were insufficient for the PCR and the presence of a few irrelevant PCR fragments in each case. Therefore, JSK04-Eh-V infection that occurred in the zoo was presumed to have been spread by a single strain because the spread of infection occurred in limited primate groups within a particular zone of the zoo at around the same time. The route of transmission of the infection from the isolated group of primates in captivity, including individuals infected with JSK04-Eh-V, to the other groups was not clear. We are now considering the possibility of cysts as the causative agents of JSK04-Eh-V infection because E. histolytica cysts have been reported to be capable of surviving and retaining their infectivity for a month under appropriate wet conditions.[17]

The symptoms of the zoo primates infected with JSK04-Eh-V differed considerably depending on their species—the symptoms in the De Brazza's guenon were relatively mild and those in the 2 individuals of Abyssinian colobus monkey and Geoffroy's spider monkey were severe and fatal. There may appear to be species-specific differences among the primates with regard to susceptibility. Although the transmission route was not clear, it is possible that the primates may be cyst carriers and sources of the contagion. Prior to this study, JSK04-Eh-V infection was thought to have been eradicated owing to the diligence of the veterinarians and zookeepers working in the zoo, and fortunately, no zoonotic infection including amoebiasis was found among the zookeepers.

YIMDHA-S was designed for the axenic culture of E. histolytica, JSK04-Eh-V, and E. dispar. This medium is considered to be efficient in comparing biological characteristics of JSK04-Eh-V with E. histolytica and E. dispar, such as the intensity of in vitro virulence to mammalian tissue culture cell lines,[9] in a single medium under the same culture conditions.