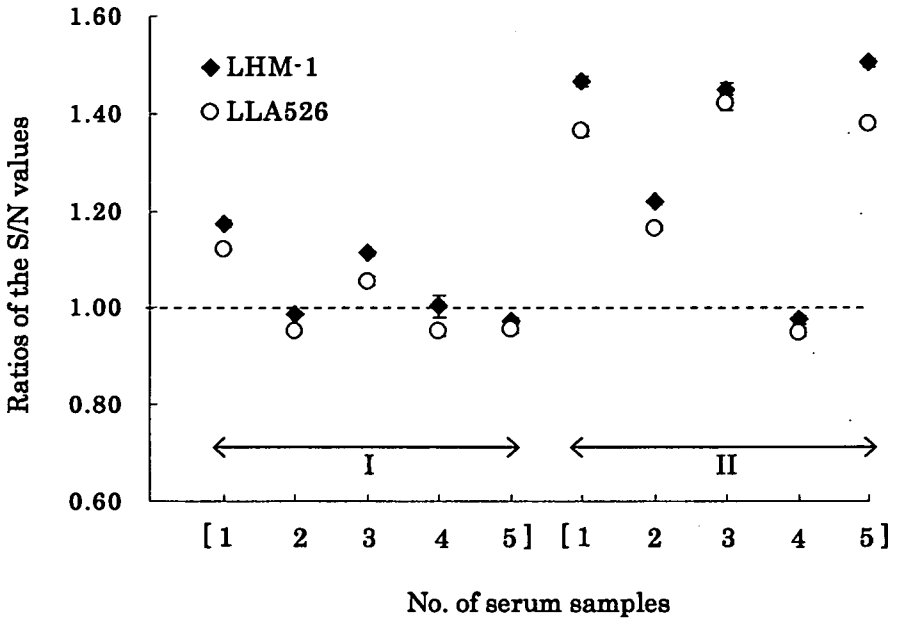

Figure 1

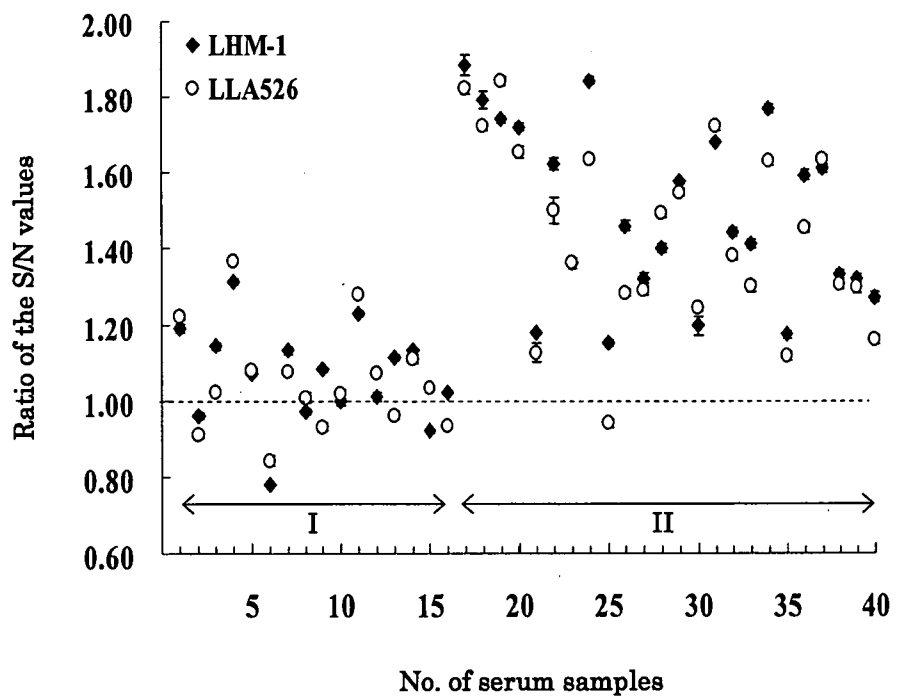

Figure 2

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.

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CAPTIONS

Figure 1. PCR products of the DNA samples from Entamoeba histolytica, JSK2004 type E. histolytica-like variant (JSK04-Eh-V), and Entamoeba dispar differentiated by multiplex PCR.
Lane 1: mixture of the DNA templates of lanes 2, 3, and 4; Lane 2: DNA of HM-1:IMSScl6 (E. histolytica; length, 475 bp); Lane 3: DNA of JSK2004 clone 2 (JSK2004cl2; JSK04-Eh-V; length, 848 bp); Lane 4: DNA of SAW1734RclAR (E. dispar; length, 195 bp); Lane 5: Human E. histolytica DNA sample (pus from liver abscess); Lane 6: Human E. histolytica DNA sample (stool); Lane 7: JSK04-Eh-V DNA sample (pus from liver abscess) from a Geoffroy's spider monkey; Lane 8: JSK04-Eh-V DNA sample (stool) from a De Brazza's guenon; Lane 9: Human E. dispar DNA sample (stool); and Lane 10: E. dispar DNA sample (stool) from a De Brazza's guenon

Figure 2. Differentiated PCR products of the DNA samples of Entamoeba chattoni and Entamoeba hartmanni using PCRs.
Lane 1: The product (length, 215 bp) from an E. chattoni DNA sample (stool) from a mandrill; Lane 2: The product (length, 215 bp) from an E. chattoni DNA sample (stool) from a Japanese macaque; Lane 3: The product (length, 423 bp) from an E. hartmanni DNA sample (stool) from a Japanese macaque.

Figure 3. Comparison of the chitinase sequences of the E. histolytica-like variant strain JSK2004cl2 and E. histolytica-like variants detected from Ateles geoffroyi and Pithecia pithecia with the sequences of the reference E. histolytica-like variant strain isolated from Macaca fascicularis
JSK2004cl2: The sequence of the chitinase gene of DNA from JSK2004cl2 obtained from a De Brazza's guenon (GenBank accession no. AB426705).
*Samples: The chitinase gene sequences of DNA samples from a Geoffroy's spider monkey (1 pus sample from a liver abscess) and white-faced saki (2 stool samples); EHMfas1: The chitinase gene sequences (GenBank accession no. AB282755) of the DNA of an isolate of another type of E. histolytica-like variant isolated from a cynomolgus monkey.[15]

Figure 4. Comparison of the sequences in the SREHP of the E. histolytica-like variant JSK2004cl2 strain with the reference E. histolytica-like variant strains isolated from Macaca fuscicularis and Macaca mulatta

JSK2004cl2: The sequence of the serine-rich E. histolytica protein (SREHP) gene in the DNA of JSK2004cl2 from a De Brazza's guenon (GenBank accession no. AB426706); EHMfas1: The sequences of the SREHP gene (GenBank accession no. AB197935) in the DNA of an isolate of another type of E. histolytica-like variant isolated from a cynomolgus monkey;[15] P19: The sequence of the SREHP gene (GenBank accession no. AB282662) in the DNA of another type of E. histolytica-like variant (an established strain) isolated from a rhesus monkey.[13]

Figure 5. The growth kinetics of the axenically cultured HM-1:IMSScl6, JSK2004cl2, AS 16 IR, and CYNO 09:TPC strains in the YIMDHA-S medium.

The mean numbers of amoebae in duplicate cultures have been plotted. YIMDHA-S: Yeast extract-iron-maltose- dihydroxyacetone-serum

Table 2. The results of the surveillance for the prevalence of the JSK2004 type Entamoeba histolytica-like variant (JSK04-Eh-V) by a newly designed multiplex PCR and microscopic examination in a zoo of Tokyo, Japan

Primate species	Common name	Number of samples	Positive number by multiplex PCR (Symptoms)			Antigen detection[a]	Microscopic examination
			E. histolytica	JSK04- Eh- V	E. dispar		
Old world monkeys:							
Cercopithecus neglectus	De Brazza's guenon	3	0	1 (Asymptomatic)	2	1	E. coli (3), E. nana (2)
Colobus guereza	Abyssinian colobus	11	0	2 (ALA[c]; Asymptomatic)	2	1	E. coli (8), E. nana (3), G. intestinalis (4)
Macaca fuscata	Japanese macaque	12	0	0	2	0	E. chattoni (7), E. coli (2), E. hartmanni (5)
Mandrillus sphinx	Mandrill	2	0	0	0	0	E. chattoni (1), E. nana (1)
Lemur catta	Ring-tailed lemur	2	0	0	0	0	G. intestinalis (2)
Varecia variegata	Ruffed lemur	2	0	0	0	0	-
Nycticebus pygmaeus	Lesser slow loris	1	0	0	0	0	-
New world monkeys:							
Aotus trivirgatus	Northan night monkey	1	0	0	0	0	C. mesnili (1)
Ateles geoffroyi	Geoffroy's spider monkey	7	0	1 (Colitis)	1	0	-
Pithecia pithecia	White-faced saki	5	0	2 (ALA[c]; Asymptomatic)	1	2	G. intestinalis (1), E. coli (1), E. nana (1)
Saguinus oedipus	Cotton-top tamarin	1	0	0	0	0	-
Total		47	0	6	8	4	

Table 1. Oligonucleotide primers used for PCR assays in present study

Primer name		Primer sequence (5' to 3')	Nucleotide position	Accession no.
EnthF	(forward)	ATG GCC AAT TCA TTC AAT GA	198-217	X65163
EnthR	(reverse)	TAC TTA CAT AAA GTC TTC AAA ATG T	648-672	X65163
EhvF	(forward)	ATT TTA TAC ATT TTG AAG ACT TTG CA	642-667	AB426549
EhvR	(reverse)	CTC TAA CCG AAA TTA GAT AAC TAC	1466-1489	AB426549
EhvR2	(reverse)	CAG ATT AAG AAA CAA TGC TTC TTC	1052-1075	AB426549
EntdF	(forward)	GTT AGT TAT CTA ATT TCG ATT AGA AC	1467-1492	AB282661
EntdR	(reverse)	ACA CCA CTT ACT ATC CCT ACC TA	1639-1661	AB282661
EchatF	(forward)	AGG ATT TGT TTT ATA ACA AGT TC	471-493	AF149912
EchatR	(reverse)	AAT AAC CTT TCT CCT TTT TCT ATC	660-685	AF149912
EhartF	(forward)	GTG AAG AGA AAG GAT ATC CAA AGT	221-244	AF149907
EhartR	(reverse)	ATA TCA TTT TCA ACT ACG AGC	623-643	AF149907
Chitinase	(forward)	GGA ACA CCA GGT AAA TGT ATA	466-487	U78319
Chitinase	(reverse)	TCT GTA TTG TGC CCA ATT	799-817	U78319
SREHP	(forward)	GCT AGT CCT GAA AAG CTT GAA GAA GC	258-286	M80910
SREHP	(reverse)	GGA CTT GAT GCA GCA TCA AGG T	784-806	M80910
R1 (locus1-2)	(forward)	CTG GTT AGT ATC TTC GCC TGT	1-21	AF276055
R2 (locus1-2)	(reverse)	CTT ACA CCC CCA TTA ACA AT	383-401	AF276055

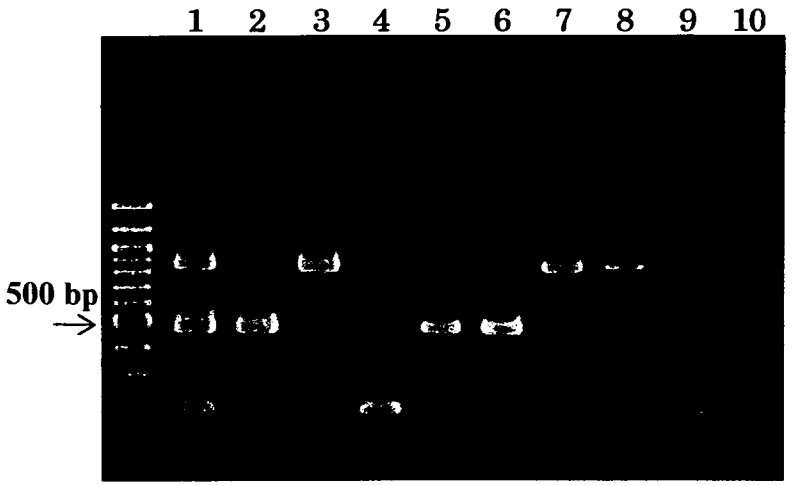

Figure 1.

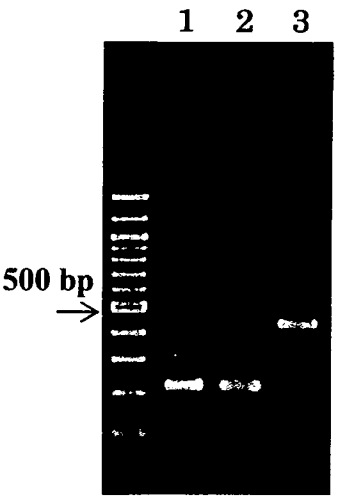

Figure 2.

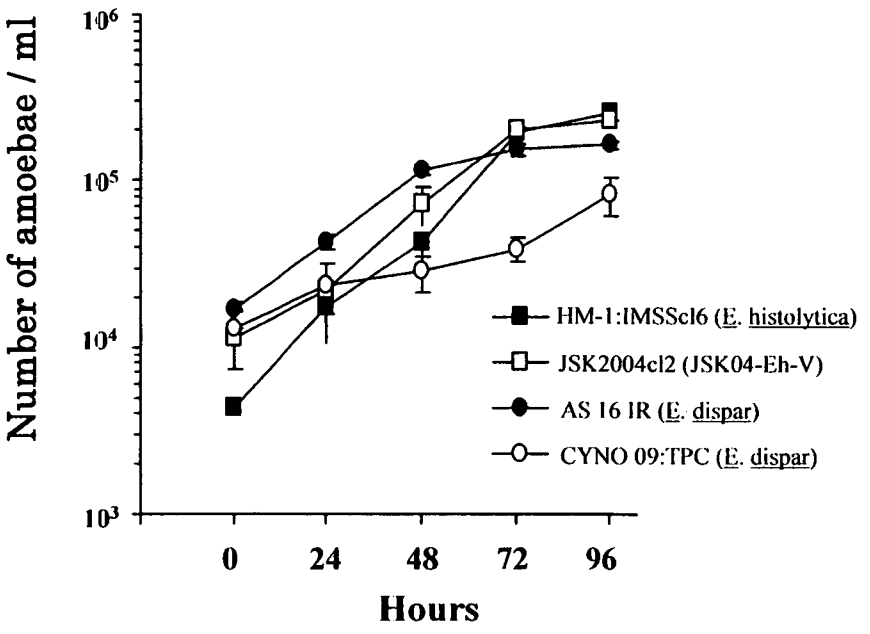

Figure 5.

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JSK2004c12 GGA ACA CCA GGT AAA TGT ATA GGA GAA ACT GTT TGT AAA TGT GGC AGA ACA CAA TAT AAC
Samples*   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
EHMfas1    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

JSK2004c12 CCT TGT GTG TGG AAT TTC CTT GAC CTT CCT GAT TGT GAA AAA AAG CCA GGT GAT TTC TTT
Samples    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
EHMfas1    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

JSK2004c12 GAG AAG TCA CCA GAT TCT TCT GAA TCT AAG CAT GAA TCT TCT GAA ATT AAA CCA GAT TCT
Samples    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
EHMfas1    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... --- --- --- --- --- ---

JSK2004c12 TCT GAA TCT AAA CAT GAA TCT TCT GAA GTT AAA CCA GAC TCT TCT GAA TCT AAA CAT GAA
Samples    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
EHMfas1    --- --- --- --- --- --- --- --- ... ... ... ... ... ... ... ... ... ..G ... ...

JSK2004c12 TCT TCT GAA GTT AAA CCA GAT TCT TCT GAA TCT AAG CAT GAA TCT TCT GAA GTT AAA CCA
Samples    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
EHMfas1    ... ... ... A.. ... ... ..C ... ... ... ... ..A ... ... ... ... ... A.. ... ...

JSK2004c12 GAC TCT TCT GAA TCT AAA CAT GAA TCT TCT GAA ATT AAA CCA GAC TCT TCT GAA TCT AAA
Samples    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
EHMfas1    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

JSK2004c12 CAT GAA TCT TCT GAG CCA GAA GTT AGT GTC CCA AAG AAA ACA GTT GCT TAT TAT ACT AAT
Samples    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
EHMfas1    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

JSK2004c12 TGG GCA CAA TAC AGA AG (GenBank:AB426705)
Samples    ... ... ... ... ... .. 
EHMfas1    ... ... ... ... ... .. (GenBank:AB282755)
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Figure 3.

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JSK2004c12 AAG AAA AAG AAA AAA GTA GCT CAG CAA AAC CAG AAT CAA GTT CAA ACA AAG ATA ATG AAG
EHMfas1    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
P19        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

JSK2004c12 ATG AGG AAG ATG AAG ATG AAG ATG ATG AAG AAG ATG AAG ATG AGA ATG AAA AAG CAA GTT
EHMfas1    ... .-- --- -G. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
P19        ..A .-- --- -.. ... ..A .T. .A. ... .G. ... ... ... ... ... ... ... ... ... ...

JSK2004c12 CAA GTG ATA AAT CAG AAG CAA GTT CAA GTG ATA AAT CAG A-- --- --- --- -AT CAA GCT
EHMfas1    ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ... .TA ACA AAC CAG A.G ... ...
P19        ... ... ... ... ... ... ... ... .C. ... ... ... ... ..C ... .-- --- --- --- -.G ... .T.

JSK2004c12 CAA ATG ATA AAT CAG AAT CAA GCT CAA ATG ATA AAC CAG AAT CAA GCT CAA ATG ATA AAC
EHMfas1    ... G.. ... ..C ... ..G ... ... ... G.. ... ... ... ..G ... .T. ... G.. ... ...
P19        ... G.. ... ..C ... ..G ... .T. ... G.. ... ... ... ..G ... .T. ... G.. ... ...

JSK2004c12 CAG AAG CAA GTT CAA GTG ATA AAT CAG AAG CAA GTT CAA GTG ATA AAC CAG ATA ACA AAC
EHMfas1    ... .-- --- --- --- -.A .C. ..C ... ... ... ... ... ... ... ... ... ... ... ...
P19        ... .-- --- --- --- -.A .C. ..C ... ... ... ... ... ... ... ... ... ... ... ...

JSK2004c12 CAG AAG CAA GTT CAA GTG ATA AAC CAG ATA ACA AAC CAG AAG CAA GTT CAA GTG ATA AAC
EHMfas1    ... ... ... .C. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
P19        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .C. ... ... ...

JSK2004c12 CAG ATA ACA AAC CAG AAG CAA GTT CAA GTG ATA AGC CAG ATA ACA AAC CAG AAG CAA GCT
EHMfas1    ... ... ... ... ... ... ... .C. ... ... ... .A. ... ... ... ... ... ... ... ...
P19        ... ... ... ... ... ... ... .C. ... ... ... .A. ... ... ... ... ... ... ... .T.

JSK2004c12 CAA CTA ATA AAC CAG AAG CAA GTT CAA CTA ATA AAC CAG AAG CAA GTT CAA CTA GTA ATT
EHMfas1    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
P19        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

JSK2004c12 CAA -TG ATA AAT CAG AAA GTA GTT CAG ATA ATG ATA ATA ATA (GenBank:AB426706)
EHMfas1    ... A.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... (GenBank:AB197935)
P19        ... A.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... (GenBank:AB282662)
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Figure 4.

赤痢アメーバの抗原検出法

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