Table 2 Chromosomes 1 and 2 resistance gene candidates

Gene	Description	B6 (mean signal intensity, relative units)	CBA (mean signal intensity, relative units)	Fold change (CBA/B6)	Location	Affymetrix probe set	P
C57BL/6J upregulated resistance candidates							
Ctdspl2[a]	(Sequences not annotated)	128.93	0.17	−755.56	2 E5|2 69.0 cM	1442424_at	0.019
Trp53bp1[a]	Transformation related protein 53 binding protein 1	141.63	0.28	−501.74	2 E5	1442316_x_at	0.019
Spata5l1[a]	Spermatogenesis associated 5-like 1	130.76	8.79	−14.87	2 E5	1455863_at	0.0023
LOC620009 /// Pdhb	Pyruvate dehydrogenase (lipoamide) beta	227.26	23.3	−9.75	1 A1/14 A1	1448214_at	0.00085
Arfgef1	(Sequences not annotated)	170.69	41.26	−4.14	1 A2	1444175_at	0.0045
Ckap2 l	RIKEN cDNA 2610318C08 gene	464.55	117.19	−3.96	2 F1	1435938_at	0.023
Hisppd2a	RIKEN cDNA B430315C20 gene	220.15	69.93	−3.15	2 E5	1442466_a_at	0.000394
Tmem127	RIKEN cDNA 2310003P10 gene	951.3	381.05	−2.5	2 F1|2 62.4 cM	1433895_at	0.0085
Pdrg1	RIKEN cDNA 1110004D19 gene	534.52	225.67	−2.37	2 H1	1419933_at	0.014
Ly96[a]	Lymphocyte antigen 96	266.78	121.91	−2.19	1 A3	1449874_at	0.0024
Bcl2l11[a]	BCL2-like 11 (apoptosis facilitator)	820.09	377.97	−2.17	2 F3-G1	1456005_a_at	0.049
Cspp1	RIKEN cDNA 4930413O22 gene	363.26	178.12	−2.04	1 A2	1431405_a_at	0.025
Spred1	Sprouty protein with EVH-1 domain 1, related sequence	258.97	139.93	−1.85	2 E5	1423162_s_at	0.000999
Zfp106	Zinc finger protein 106	633.56	348.89	−1.82	2 F1|2 67.2 cM	1425331_at	0.0048
Rin2	15 days embryo head cDNA, RIKEN full-length enriched library, clone:D930006C05 product:unknown EST, full insert sequence	318.72	183.74	−1.73	2 G1	1441209_at	0.000507
Cds2	CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 2	1834.58	1070.35	−1.71	2 F2|2 73.0 cM	1438957_x_at	0.027
1200015F23Rik	RIKEN cDNA 1200015F23 gene	1043.3	613.11	−1.7	2 E5	1428196_a_at	0.045
Sord	sorbitol dehydrogenase 1	860.62	546.68	−1.57	2 E5|2 66.0 cM	1426584_a_at	0.015
Epb4.1l1	Erythrocyte protein band 4.1-like 1	277.83	177.64	−1.56	2 H1|2 88.0 cM	1434575_at	0.024
Smox	Spermine oxidase	378.4	248.34	−1.52	2 F1	1424268_at	0.012
CBA/J upregulated susceptibility candidates							
Tmem87a[a]	RIKEN cDNA A930025J12 gene	88.19	975.22	11.06	2 E5	1424454_at	0.0014
Pldn[a]	(Sequences not annotated)	57.07	424.56	7.44	2 E5|2 67.6 cM	1457088_at	0.00027
Actc1	actin, alpha, cardiac	699.32	2084.01	2.98	2 E4|2 64.0 cM	1415927_at	0.0087
Eid1	CREBBP/EP300 inhibitory protein 1	522.35	1451.48	2.78	2 F1	1448406_at	0.019
Adal	RIKEN cDNA 4930578F03 gene	122.78	309.83	2.52	2 F1	1428198_at	0.018
BC052040	10 days neonate cerebellum cDNA, RIKEN full-length enriched library, clone:B930089B11 product:unknown EST, full insert sequence	79.86	182.11	2.28	2 E4	1460144_at	0.0011
Myl9	Myosin, light polypeptide 9, regulatory	2767.14	5264.28	1.9	2 H1	1452670_at	0.0089
Rbm39	Expressed sequence C79248	1448.29	2726.52	1.88	2 H1	1446147_at	0.020

[a]Gene dysregulation validated by qRT-PCR (Supplementary Figure 1).

protective or deleterious during amebiasis. In a SCID mouse model of amebic liver abscess, hepatocyte apoptosis plays a pathologic role in abscess formation.[28] In contrast in intestinal helminth models epithelial apoptosis is thought to be protective by maintaining crypt length and epithelial homeostasis.[29]

Beyond these chromosomes 1 and 2 loci that associate with resistance to the establishment of infection, the presence of other loci that associate with parasite burden once infected (chromosome 13) and the resultant inflammatory response (chromosome 9) suggest the presence of multiple layers of host control in amebiasis. The LRS scores for these loci were modest and should not be overinterpreted in the absence of confirmatory evidence in congenic or recombinant inbred strains. However the concept of sequential nodes of host control is in keeping with our working understanding of this model, which holds that amebic infection proceeds through multiple phases, step one being parasite survival amid the epithelium and step two being survival amid the inflammatory response. Therefore the possibility of distinct genetic loci that associate with these two steps was anticipated. As the mouse genome becomes annotated and whole genome association studies unfold from human amebiasis sites[9] it is hoped that findings such as these can be directly tested to shed light on the pathogenesis of this disease.

Materials and methods

Mice

C57BL/6, CBA/J, B6CBAF$_1$ (B6 × CBA) and BXH14/TyJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). FVB mice were obtained from Craig Coopersmith (Washington University, St Louis, MO, USA). Muc2 KO mice were obtained from Anna Velcich (Albert Einstein Cancer Center/Montefiore Medical Center, New York, NY, USA) and tested against wild-type littermates. N_2 mice were bred by backcrossing B6CBAF$_1$ female with CBA/J male mice. Animals were maintained under specific pathogen-free conditions at the University of Virginia and were challenged at 4–8 weeks of age. The Institutional Animal Care and Use Committee approved all protocols. Recombinant inbred strain BXH14/TyJ contains unique, approximately equal proportions of genetic contributions from two progenitor C3H/HeJ and C57BL/6J inbred strains and is, importantly, homozygous for C3H/HeJ at whole chromosome 1 and the latter half of chromosome 2 (http://www.well.ox.ac.uk/mouse/INBREDS/RIL/BXH.shtml).

Parasites and intracecal inoculation

Trophozoites for intracecal injections were originally derived from laboratory strain HM1:IMSS (American Type Culture Collection, Manassas, VA, USA) that have been sequentially passaged *in vivo* through the mouse cecum. Cecal contents were cultured in trypsin-yeast-iron (TYI-S-33) medium supplemented with 25 U ml^{-1} penicillin and 25 mg ml^{-1} streptomycin. For all intracecal inoculations, trophozoites were grown to the log phase, counted with a hemacytometer and 2×10^6 trophozoites in 150 µl were injected thrice intracecally after laparotomy as described.[10] For select experiments dexamethasone was administered to mice 0.2 mg i.p. on days −3, −2, −1 and 0 to enhance susceptibility of CBA mice. For cecal wash experiments, cecal mucinous intestinal washes were prepared by flushing cecal contents with completed TYI media and centrifuging at 14000 *g* × 10 min (simplified from Carlstedt *et al.*[30]). Supernatant was incubated 1:1 with an *E. histolytica* pellet of 2×10^6 trophozoites at 37 °C for 1 h and ameba were then injected intracecally.

Pathology and scoring of amebic colitis

Mice were killed and each cecum longitudinally bisected. One half of the cecum was placed in Hollande's fixative, cut into 3–5 equal cross sections, paraffin embedded and 4 mm sections were stained with hematoxylin and eosin. Histopathology was scored in blinded fashion for inflammation score and ameba score for each mouse as described previously.[16] To determine infection rate by culture, the contents of the other half of the cecum were rinsed in 1 ml phosphate-buffered saline (PBS) and cultured in TYI-S-33 medium. To determine infection severity 200 µl of this material was tested for *E. histolytica* antigen by ELISA (*E. histolytica* II test, Techlab, Blacksburg, VA, USA). ELISA values were normalized for background and positive control optic density.

Lectin histochemistry

Purified *E. histolytica* Gal/GalNAc lectin was prepared as previously described.[14] Frozen acetone-fixed sections of cecal tissue were stained with or without 20 µg lectin per ml × 30 min at room temperature, followed by 12 µg ml^{-1} rabbit polyclonal anti-*E. histolytica* Gal/GalNAc lectin antibody × 30 min at room temperature, followed by biotinylated goat anti-rabbit immunoglobin G, ABC working solution and diaminobenzidine (Vector, Burlingame, CA, USA) as per the manufacturer's instructions. The identical protocol without Gal/GalNAc lectin was performed as control.

Genotyping

Genomic DNA was isolated from the tails and livers of mice by using the standard phenol/chloroform extraction and ethanol precipitation method. A total of 150 markers with 32 microsatellites and 118 single nucleotide polymorphisms, distinguishing B6 from CBA strain and covering all 19 autosomes and the X chromosome, were used to identify which of the two parental strains contributed to alleles at a specific locus of each N_2 mouse (Table 1). Parental and F_1 DNA served as control for each marker. The linkage maps and marker positions reported were determined using our own data and Map Manager.

Epithelial microarrays

Affymetrix gene chip analysis was performed according to the manufacturer's instructions using murine MOE430 2.0 arrays. Full public access to the raw data is available at https://genes.med.virginia.edu/public_data/index.cgi under 'Eric_Houpt_CBAvsB6naive'. Information about a microrarray experiment (www.mged.org/miame) includes the following. Ceca were obtained from male 6-week-old CBA/J and B6 mice ($n = 3$ each). Cecal contents were rinsed in sterile PBS and the cecal wall was scored, and serosal and muscularis layers were removed. Mucosal epithelial tissues were then placed in RNAlater (Ambion, Austin, TX, USA), homogenized and total RNA extracted using the RNAEasy kit (Qiagen, Valencia, CA, USA). Ribosomal peaks were intact and showed no

evidence of degradation. The BioB control cRNA was spiked in at the detection threshold of 1.5 pM and received a 'present' detection call. Housekeeping genes had 3′–5′ detection ratios of <4. Complementary DNA synthesis, *in vitro* transcription to cRNA and hybridization were performed as per http://www.healthsystem.virginia.edu/internet/biomolec/microarray.cfm.

Raw data from the arrays was normalized at probe level using gcRMA algorithm. The detection call (Present, Marginal, Absent) for each probe set was obtained using the GeneChip Operating Software system. CBA/J vs B6 groups were compared using dChip software, whereby gene expression was deemed significantly different if the *P*-value was less than or equal to 0.05, the fold change in signal intensity was >1.75 or <−1.75, and the absolute difference in signal intensity was >100.

RT-PCR

Quantitative RT-PCR was used to validate changes in a subset of genes from microarray selection from each of the three CBA and three B6 RNA samples. RT-PCR primers were designed using Primer3 based on the published 3′ sequence as per Affymetrix. The primers used were glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*): forward, GCTAAGCAGTTGGTGGTGCA and reverse, TCACCACCATGGAGAAGG; BCL2-like 11 apoptosis facilitator (*Bcl2l11*): forward, TCCTATTTCC TTCCAGTGTTGT and reverse, GGGAACACAATTGCA CAGAG; lymphocyte antigen 96 (*Ly96*): forward, CTTTTCGACGCTGCTTTCTC and reverse, CCATGGC ACAGAACTTCCTT; transformation related protein 53 binding (*Trp53bp1*): forward, AGTCCTGAGACAGA GGCCAGT and reverse, GTGTGGGTGCCACAGAGGT; transmembrane protein 87A (*Tmem87a*): forward, TGT GGTGCACACTCCAAAA and reverse, TAAGCTCCCC TTCTGAAGCA; pallidin (*Pldn*): forward, GCAGCTCA CAGCCATCTGTA and reverse, TTAGGGCTTCATCA CCTATGG; carboxy-terminal domain, RNA polymerase II, polypeptide A (*Ctdspl2*): forward, GAGAGCCCAAT GATTAGCAA and reverse, CAGCAACAAAATAATTA TAAAGTCAGA; spermatogenesis associated 5-like 1 (*Spata5l1*): forward, CGGCCTGGAAGATTAGATAAGA and reverse, CACAGTCTTGAGTGCTTCTGTG. GAPDH was used as the internal control. Real-time PCR was conducted using iQ SYBR Green SuperMix (Bio-Rad, Hercules, CA, USA) on an Bio-Rad iCycler. Target gene amplification was quantified as the reciprocal $\log_2$ of the $\Delta C(t)$ between GAPDH and the target gene.

Statistics

Genetic markers were assigned to and mapped within the chromosomes using multipoint linkage analysis with Map Maker QTXb20. Genome-wide interval mapping for amebic antigen load measured by ELISA and inflammation score was performed using QTX software to identify QTLs. All QTL mapping methods offered by QTX generate a LRS as a measure of the significance of a possible QTL.[31] The LRS approaches a χ^2-statistic with large sample sizes. Single-locus associations were tested by simple regression analysis between trait values and genotypes and the significance of each potential association was measured using the LRS. A total of 5000 permutations of the trait values were used to define the genome-wide LRS threshold required to be significant or suggestive for each specific trait. Loci exceeding the 95th percentile of the permutation distribution were defined as significant ($P<0.05$) and those exceeding the 37th percentile were suggestive ($P<0.63$).[32] Statistical significance for infection rate was determined by Fisher's exact test. Means were compared using *t*-test or Mann–Whitney test if data were not gaussian. RT-PCR differences were determined using unpaired Student's *t*-test. Fold changes between RT-PCR and microarray were compared using linear regression analysis using Pearson's R correlation.

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Disclosures

The authors have no conflicting financial interests.

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Mini-Review

Plants, endosymbionts and parasites

Abscisic acid and calcium signaling

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Key words: parasite, calcium, signaling, hormone, stress, chemotherapy

It was recently discovered that the protozoan parasite, *Toxoplasma gondii* produces and uses the plant hormone, abscisic acid (ABA), for communication. Following intracellular replication, ABA production influences the timing of parasite egress from the host cell. This density-dependent signal may serve to coordinate exit from the host cell in a synchronous manner by triggering calcium-dependent activation of motility. In the absence of ABA production, parasites undergo differentiation to the semi-dormant, tissue cyst. The pathway for ABA production in *T. gondii* may be derived from a relict endosymbiont, acquired by ingestion of a red algal cell. Although the parasite has lost the capacity for photosynthesis, the plant-like nature of this signaling pathway may be exploited to develop new drugs. In support of this idea, an inhibitor of ABA biosynthesis protected mice against lethal infection with *T. gondii*. Here, we compare the role of ABA in parasites to its activities in plants, where it is know to control development and stress responses.

Toxoplasma gondii: Ubiquitous Protozoan Parasite with a Plant-like Endosymbiont

T. gondii is found in a wide range of warm-blood animals and infects about one third of world's human population.[1] While normally benign, infections in immunocompromised patients can result in severe disease.[1] This parasite has two main proliferative stages that contribute to infection in humans.[2] Tachyzoites replicate rapidly within a segregated vacuole, rupture out of the cell, and re-invade new cells. In contrast, bradyzoites encyst and grow slowly, mainly in long-lived cells of the central nervous system and musculature. The encysted form can survive for a long time and poses a risk due to reactivation following host immuno-suppression. Once reactivated, parasites revert to the lytic phase characterized by rapid growth, egress and invasion of new host cells, thus causing considerable tissue damage.

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T. gondii is a parasite in the phylum Apicomplexa, which includes a large number of obligate intracellular parasites. The causative agent of malaria, *Plasmodium* and a cause of serious gastrointestinal disease, *Cryptosporidium*, also belong to the Apicomplexa. Most apicomplexans contain a special organelle called the apicoplast, which was acquired by engulfment of an algal cell in a secondary endosymbiotic event.[3,4] The original endosymbiont likely had four membranes; two of them derived from photosynthetic bacteria and two additional ones from the algal cell and progenitor host cell plasma membranes.[5] This configuration persists in *T. gondii* (Fig. 1A), although some reports indicate only three membranes surround the malaria apicoplast.[5] Debate about the origin of this endosymbiont was recently resolved by the discovery of a photosynthetic relative of the Apicomplexa and their sister taxa the dino a new flagellates. Prototrophic branch of the tree is represented by the marine alga called *Chromera*, which contains an endosymbiont of red algal origin that retains key features seen in Apicomplexa and dinoflagellates.[6] Since its engulfment, the endosymbiont has undergone a variety of rearrangements in different derived lineages to shuffle most of the genes to the nucleus. In the process, many pathways have been modified or entirely lost, for example the absence of photosynthesis among all apicomplexans.[5]

The presence of the apicoplast implies that *T. gondii* and related parasites have the potential to use plastid or algal-like pathways derived from the endosymbiont. Previous studies have highlighted the importance of various pathways for metabolism such a fatty acid FAS II-like pathway, ferridoxin-ferrodoxin-NADP$^+$ reductase, heme biosysnthesis and a DOXP isoprenoid biosynthesis pathway.[5] These have been suggested as important targets for development of new therapeutics, which is especially attractive given their bacterial or plastid-like nature. However, acquisition of primitive signaling pathways had not been considered previously. Thus, the discovery of the ABA response pathway in *T. gondii* is notable as it suggests early branching alga may have contained metabolites that control signaling between cells, and these pathways may have been inherited in parasites.

Calcium, Motility and Signaling in *T. gondii*

T. gondii uses intracellular calcium as a signal to control protein secretion and to activate motility, two events that are crucial to the parasite's intracellular existence.[7] Apicomplexan parasites undergo

actin-based gliding on substrates and this form of motility powers cell invasion.[8] Intracellular calcium stores are required for activating increases in cytosolic calcium, which induces protein secretion and facilitates cell invasion.[9] Intracellular calcium has also been implicated in controlling egress of the parasite from infected cells.[10] Pharmocological evidence suggests the presence of both ryanodine-responsive and IP_3-receptor-like channels, although the genes encoding these activities have not been identified in apicomplexan parasites.[11] In searching the genome for calcium regulation pathways, it became clear that *T. gondii* lacks many animal-like pathways, yet has affinities with plants.[12] For example, *T. gondii* lacks conventional IP_3 channels but instead contains a gene that is similar to the two-pore calcium channel found in plants.[12,13] Apicomplexans also contain a variety of calcium-dependent protein kinases, which are highly developed in plants but absent in animals.[14]

The second messenger cyclic ADP ribose (cADPR) evokes changes in cytosolic calcium in both plants[15] and animals[16] by stimulating calcium release from intracellular stores. *T. gondii* also uses the second messenger cADPR to stimulate release of internal calcium stores.[17] The antagonist 8-Br-cADPR inhibits protein secretion and motility in the parasite,[17] indicating this pathway is important for controlling cell invasion. Plants generate cADPR in response to the phytohormone ABA,[18] leading to elevated cytosolic calcium that controls stomata closure in guard cells and induces gene expression.[18] ABA has also been linked to generation of cADPR and induction of calcium signaling in other metazoans including hydra[19] and sponges.[20] Recently, human granulocytes (myeloid cells) were found to produce ABA, which triggers calcium increases by a cADPR-dependent pathway.[21] This prompted us to consider whether apicomplexan parasites might also contain such an ABA-dependent, cADPR pathway for controlling calcium release.

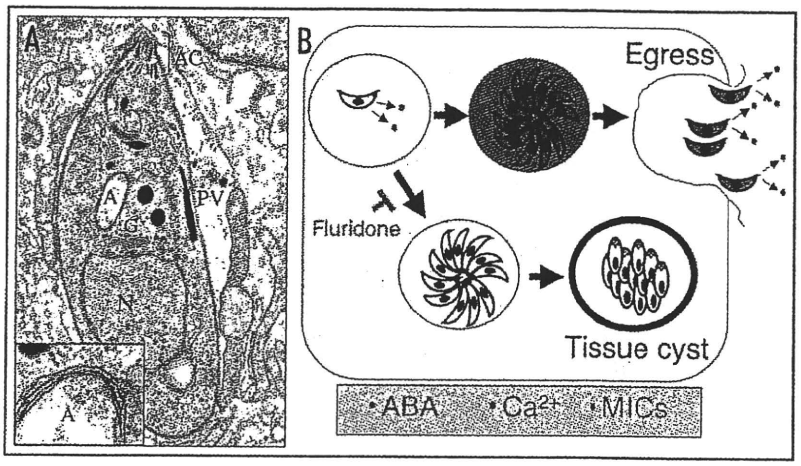

Figure 1. ABA production in *T. gondii*. (A) Cross-section through an intracellular *T. gondii* showing organellar structures. Insert illustrates four surrounding membranes from enlarged apicoplast. N, nucleus; A, apicoplast; AC, apical complex consisting of conoid and secretory organelles; PV, parasitophorous vacuole. (B) Model of ABA production by the parasite with in the PV. Accumulation of ABA (pink) leads to increases in intracellular calcium (blue) activating egress and triggering secretion of micronemes (MICs shown in green). Fluridone blocks ABA production, preventing egress and favoring differentiation of tissue cysts.

ABA Production and Signaling in *T. gondii*

Addition of exogenous ABA to extracellular *T. gondii* parasites strongly stimulated secretion of proteins from apical organelles called micronemes,[22] a process necessary for parasite motility and cell invasion. Previous studies have shown that this pathway relies on intracellular calcium and is mediated by increases in cADPR.[11,17] Addition of ABA also stimulated increases in cADPR and lead to a calcium-dependent induction of protein secretion.[22] This activity was restricted to (± ABA) and was not stimulated by β-carotene, retinoic acid or (-) ABA.[22]

Further evidence that *T. gondii* contains ABA was provided by ELISA using an antibody specific to ABA and by mass spectrometric (MS) analysis.[22] Levels of ABA reached a peak just prior to natural egress, when parasite-containing vacuoles reach their maximum size. MS/MS analysis revealed the characteristic fragments for both *trans*-ABA and *cis*-ABA in extracts of *T. gondii* that were subjected to HPLC purification and gas chromatography and tandem MS/MS analysis.[22] The levels of ABA in parasites ranged from 0.1–0.2 M during initial infection to ~4 M near the end of the infection cycle, when the density of parasites within the host cell is highest. These levels are 10–40 times higher than that reported for human granulocytes,[21] supporting the idea that ABA is produced by the parasite rather than concentrated from environmental sources. Remarkably, when exogenous ABA was added to late stage vacuoles, it induced premature egress, consistent with the idea that this signal mediates exit of the parasite from the host cell. Similar to plants, the production of ABA in *T. gondii* was inhibited by treatment with fluridone, which blocks ABA-biosynthesis at the step of phytoene desaturase[24] (Fig. 2). Disruption of ABA production by fluridone inhibited the egress of *T. gondii* from the host cell. The fluridone block was rescued by addition of exogenous ABA, supporting the idea that this signal controls nature egress. Collectively, these studies indicate that *T. gondii* produces ABA, elevating the second messenger cADPR, and increasing intracellular calcium to control motility and cell egress (Fig. 1B).

In plants, ABA also controls development by acting to increase dormancy in seeds and to prevent premature germination of embryos.[25] Transcript levels of ABA biosynthetic genes (see below) in plants are also upregulated during osmotic, cold and salt stress.[25] Thus, ABA has been recognized as a stress hormone, able to increase plant tolerance to stressful conditions. A role in the stress response does not appear to be directly analogous in parasites. Instead repression of ABA synthesis by fluridone induced the differentiation to bradyzoites in vitro.[22] Dormancy in *T. gondii* is normally a stress-induced pathway that can be triggered by nutrient limitation.[26] Thus, differentiation to bradyzoites in the absence of ABA may simply reflect a default pathway for parasites remaining within a host cell that contains diminishing resources and which lacks a signal for egress. It is also possible that reduction in ABA synthesis may make parasites more susceptible to stressful conditions in the environment, thus triggering a differentiation process that would otherwise be blocked under high levels of ABA.

Fluridone was also effective in preventing lethal toxoplasmosis in the murine model, consistent with its ability to block ABA

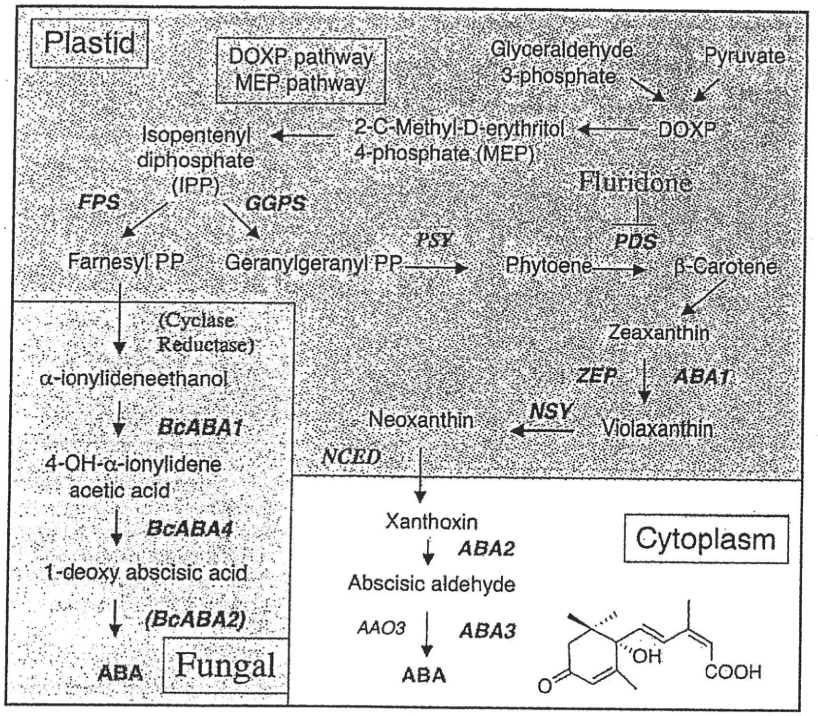

Figure 2. Biosynthetic pathways for ABA production in higher plants and fungi. This is an abbreviated scheme showing only the major steps in the pathways. For further details consult.[24,25,28] DOXP, deoxy-D-xylulose-5-phosphate; FPS, farnesyl diposphate synthase; GGPS, geranylgeranyl diphosphate synthase; PSY, phytoene synthase; PDS, phytoene desaturase; ZEP, zeaxanthin epoxidase; NSY, neoxanthin synthase; NCED, 9-cis-epoxycarotenoid dioxygenase; ABA, abscisic acid.

production, delay egress and thus prevent spread of infection.[22] Previous studies demonstrate the related compound norflorizone is also effective at inhibiting malaria in vitro,[27] although the biological role of ABA has not been studied in this system. Because fluridone is an effective herbicide that is relatively non-toxic to mammals, it may provide a good lead for the development of anti-parasitic compounds to combat infection.

Pathways for ABA Production in Plants, Fungi and Algae

The synthesis of ABA in higher plants occurs by an indirect pathway that is initiated by cleavage of carotenoids in the plastid and subsequent modification of the resulting xanthoxin products in the cytosol[24] (summarized in Fig. 2). The pathway is known from a variety of metabolic studies as well as mutants that are disrupted in specific steps of synthesis, largely from studies in Arabidopsis, maize and tomato.[24] In plastids, the β-carotene hydroxylated product zeaxanthin is converted to violaxanthin by a zeaxanthin epoxidase (ZEP), also known as *ABA1* in Arabidopsis. Mutants in this step are defective in production of ABA, demonstrating that ABA is derived from C_{40} carotenoids. The first committed step in the pathway to ABA is catalyzed by 9-*cis*-epoxycarotenoid dioxygenase (NCED), which cleaves the C_{40} substrate 9-*cis*-epoxycarotenoid to yield the C_{15} intermediate xanthoxin. NCED is related to dioxygenases in mammalian cells that generate vitamin A (retinol) from β-carotene obtained in the diet. NCED activity is impaired in the *viviparous 14* (*vp14*) mutant of maize, leading to premature seed germination. Subsequent modification of xanthoxin by ABA2, a short-chain alcohol dehydrogenase/reductase, yields abscisic aldhehyde. This product is then converted to ABA, by the action of ABA aldehyde oxidases (AAO), which require the molybdenum cofactor (MoCo) sulfurase (ABA3)-activated MoCo factor for the catalytic activity. ABA is catabolized in plants to the inactive phaseic acid by the action of ABA 8'-hydroxylases. Fungi also produce ABA; however, they use a direct route from farnesyl pyrophosphate through a cyclized C_{15} intermediate known as α-ionylideneethanol[28] (Fig. 2).

The initial steps in ABA synthesis in higher plants take place in the plastids, attesting to their origin from algal endosymbionts. While it is not yet clear if the downstream enzymes are conserved in algae, there is compelling evidence for ABA biosynthesis in these organisms. ABA has been reported in a wide variety of algae including green (chlorophyta), red (rhodophyta), brown (heterokontophyta) and several cyanobacteria, with intracellular concentrations ranging from 1~40 M.[29] Radiolabeling studies indicate that ABA production by the green alga *Dunaliella salina* is enhanced by hypersalinity stress.[30] Cyanobacteria have also been reported to produce ABA under stress conditions.[31] Furthermore, ABA influences stress responses and morphogenesis in the green alga *Haematococcus pluvialis*[32] and induces differentiation and spore formation in the brown alga *Laminaria japonica*.[31]

While there is clear evidence for the production of ABA and physiological responses to this hormone in algae, the biosynthetic pathway is less well studied than in higher plants. Moreover, higher plants often contain gene families that encode ABA biosynthetic enzymes.[24] This gene redundancy may partly contribute to the 'leaky' phenotypes of certain ABA-deficient mutants (i.e., *vp14* in maize) that are only partially impaired in ABA production.[24] Additionally although most plants contain only single *ABA2* and *ABA3* genes, residual levels of ABA in mutants of these genes may reflect alternative shunt pathways that are not fully defined. Hence, the lack of close similarity between ABA biosynthetic genes in higher plants and algae may reflect gene divergence since the common origin of plastids.[33]

Apicomplexans lack the mevalonate pathway but instead have a conserved DOXP-MEP pathway for synthesis of isoprenoids, that is thought to be derived from the plastid.[34] However, searching the *T. gondii* genome database (http://toxodb.org) does not provide strong support for a conserved carotenoid or ABA biosynthetic pathway. For example, *T. gondii* appears to lack clear orthologues for several steps in carotenoid biosynthesis including phytoene synthase (PSY) and NCED, at least based on searches using genes from higher plants. Weak orthologues for phytoene desaturase (PDS) and zeta carotene desaturase (ZDS) are found, and this is consistent with the inhibition of ABA production following treatment with fluridone.[22] *T. gondii* contains multiple candidate genes for biosynthesis of ABA (i.e., *ABA1*, *ABA2* and *ABA3*). However, these genes have low homologies with higher plant genes, and do not allow unambiguous

assignments of the enzymes in this pathway. This may reflect a divergence of the pathways in parasite and/or similarity to algal like pathways, rather than those found in higher plants. Future analysis of this pathway in algae and parasites should provide further insight into the biosynthesis pathway and the role of this hormone outside of higher plants.

Outlook

Many apicomplexans retain an endosymbiont derived from ingestion of a red algal cell, giving rise to the possibility that plastid-like pathways have been retained in these parasites. Although apicomplexans have lost the means for photosynthesis, they appear to retain a primitive pathway for production of plant hormones such as ABA. Our studies reveal that *T. gondii* produces ABA, which likely originates from the DOXP isoprenoid pathway via carotenoid intermediates. However, apicomplexans appear to lack highly conserved enzymes found in this pathway in higher plants. Whether they contain a primitive or divergent pathway, or one that resembles the direct pathway found in fungi is presently unclear. In this regard, *Chromera*, an ancestrally related photosynthetic alveolate.[6] may shed light on the pathways for carotenoid biosynthesis and downstream metabolites that existed in the ancestral algal cell that gave rise to the apicoplast.

In higher plants, the indirect pathway for ABA production begins in the plastid and is completed in the cytosol.[24] However, it is not known where ABA is synthesized, stored and accumulated in parasite-infected cells. Figure 1 shows ABA released into the vacuole; however, it is also possible it accumulates within the parasite or in the host cell. Since ABA is a weak acid (pKa 4.7) it tends to concentrate in compartments with neutral or basic pH and does not readily pass membranes. Accumulation in separate compartments may trigger different responses depending on the localization of specific mechanisms for detection.

Finally, it is not known how ABA is sensed in parasites, In this regard, the conservation of several recently described plant receptors for ABA in apicomplexan parasites is intriguing. The RNA-binding protein FCA is an ABA receptor that controls translational repression and flowering responses in plants.[35] The second of these is a G-protein-coupled receptor that functions as a plasma membrane receptor to control ABA responses in Arabidopsis.[36] Both of these receptors have putative orthologues with high BLAST hits in apicomplexan parasites (http::://ApiDB.org). Downstream of such receptors, it is also not known if ABA induces transcriptional changes in parasites, similar to those described in plants, where this is a predominant mode of action.[24,25] Thus, while our initial studies implicate ABA in calcium-triggered egress, it is likely this metabolite has many other influences on parasite biology that are yet to be discovered.

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Original Article

Seroprevalence of *Entamoeba histolytica* Infection in Female Outpatients at a Sexually Transmitted Disease Sentinel Clinic in Tokyo, Japan

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SUMMARY: From 2003 to 2006, we surveyed the seroprevalence of amoebic infection in female outpatients at a gynecologist's office, which was designated as a sexually transmitted disease sentinel clinic by the Tokyo Metropolitan Government, using an enzyme-linked immunosorbent assay (ELISA). The annual rate of anti-*Entamoeba histolytica* (HM-1:IMSScl6 strain; HM-1) antibody-positive cases as detected by ELISA increased during that period, and anti-*Chlamydia trachomatis* antibodies were detected in 60%, i.e., 24 of 40 anti-HM-1 antibody-positive individuals, suggesting sexual transmission of *E. histolytica*. We designed an ELISA with better sensitivity using the antigen extracted from the virulence-augmented *E. histolytica* strains (LHM-1 and LLA526 strains) by liver-passaging in hamsters. The average ratios of the S/N value (optical density [OD] of sample/OD of negative control) of ELISA with either the LHM-1 or LLA526 antigen and that of ELISA with the HM-1 antigen were significantly higher in intestinal amoebiasis cases with low S/N values than in amoebic liver abscess cases. In the present study of the seroprevalence of *E. histolytica* infection, the sera testing positive with low S/N values (<10) by ELISA with HM-1 antigen exhibited higher S/N values by ELISA using LHM-1 and LLA526 antigens. This modification of the antigen preparation for ELISA is expected to be effective in detecting anti-*E. histolytica* antibodies from such asymptomatic patients who have low antibody titers.

INTRODUCTION

In Japan, it was thought until the mid-1970s that amoebiasis was solely food borne and spread via food contaminated with cysts of *Entamoeba histolytica*. However, in the late 1970s, after amoebiasis was reported to have spread among men having sex with men (MSM) in large cities of the United States, it was recognized as a sexually transmitted disease (STD) (1,2). Within a few years, the suspected number of MSM having anti-*E. histolytica* antibodies along with anti-*Treponema pallidum* antibodies began to increase in densely populated cities in Japan (3,4).

In data provided by Japan's National Epidemiological Surveillance of Infectious Diseases, the number of notified cases with amoebiasis has been increasing annually; in 2006, 747 cases were reported, approximately 90% of which were male. However, with the spread of amoebiasis, the number of notified female cases has also increased at a slow but steady pace since 1999 (5,6).

In the present study, by detecting anti-*E. histolytica* (HM-1:IMSScl6 strain; HM-1) antibodies using an enzyme-linked immunosorbent assay (ELISA), we report the seroprevalence of amoebic infection in female outpatients who visited a gynecologist's office in Tokyo, Japan, from 2003 to 2006; this office was designated as an STD sentinel clinic by the Tokyo Metropolitan Government.

Moreover, in this study we attempted to design an ELISA with better sensitivity. This involved the use of the antigen extracted from the virulence-augmented *E. histolytica* strains by liver-passaging in hamsters. The serum anti-*E. histolytica* antibody titers are low in a majority of asymptomatic amoebiasis cases. Practically, this serological method using LHM-1 and LLA526 antigens was tested on the anti-HM-1 antibody-positive sera in the present surveillance study.

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MATERIALS AND METHODS

Study population: This study was conducted at a Tokyo gynecologist's office that was designated as an STD sentinel clinic by the Tokyo Metropolitan Government. We collected blood samples from 981 female outpatients between 2003 and 2006 (205 in 2003, 217 in 2004, 282 in 2005, and 277 in 2006) (Table 1). All individuals provided informed consent. Patient age was the only additional information. The anti-*E. histolytica* antibody-positive sera were examined for anti-*Chlamydia trachomatis* and anti-*T. pallidum* antibodies as indicators of STDs.

ELISA: *E. histolytica* antigens were prepared from axeni-

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
Total	205	217	282	277	981

cally cultured *E. histolytica* (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 1:8,000 diluted peroxidase-conjugated anti-human IgG rabbit serum (ICN-Cappel Inc., Aurora, Ohio, 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., St. Louis, Mo., USA; 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 *C. trachomatis* and *T. pallidum* infections: Anti-*C. trachomatis* 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 two 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 using the *T. pallidum* passive hemagglutination (TPHA) kit (Fujirebio, Inc., Tokyo, Japan) for detection of anti-*T. pallidum* 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 run of all the serological tests.

ELISA with antigens from virulence-augmented amoebae: Based on the hypothesis that the amount of antigenic substances would also decrease simultaneously with the loss of virulence, we attempted to design an ELISA with better sensitivity in the following manner: (i) 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). (ii) On the 6th day of inoculation, the hamsters were sacrificed and the livers dissected aseptically. The amoebic abscesses isolated from each of the livers were minced finely and crushed using scissors for medical use in the TYI-S-33 medium (9). (iii) 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), both *E. histolytica* 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 the significant reduction in 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 earlier. (iv) LHM-1 and LLA526 were mass cultured within 2 weeks after their transfer into TYI-S-33 medium from the amoebic liver abscesses. The antigens were then harvested and washed twice in phosphate buffered saline (PBS) by centrifugation ($175 \times g$ 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. (v) The sonicated suspensions were then centrifuged at $9,100 \times g$ for 30 min, and the protein concentrations of the aqueous soluble extracts were measured by Bradford's method (10). (vi) 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 three antigens–LHM-1, LLA526, and HM-1–and the average OD values were calculated. The sensitivity of ELISA for each of the three antigens was compared with the positive serum samples of 5 patients clinically diagnosed having amoebic liver abscesses and 5 mentally handicapped persons in a rehabilitation institution for the intellectually impaired in Japan, who were almost free from amoebiasis symptoms but positive for *E. histolytica* cysts on microscopy and for *E. histolytica* antigen when tested by using an *E. histolytica*-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 was determined 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.

RESULTS

Seroprevalence of anti-*E. histolytica* antibodies in the female population: During the 4 years 2003 to 2006, in the 981 sera samples obtained from the study population, the seroprevalence of anti-*E. histolytica* (HM-1) antibodies increased every year. In 2005 and 2006, the annual positive rate was >5%; the average annual positive rate 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-*C. trachomatis* antibodies–an indicator of STDs. On the other hand, none of the cases were positive for anti-CL antibodies (a retest by the TPHA kit was not performed). The strong positive correlation between seropositivity for anti-*E. hitolytica* and anti-*C. tracomatis* antibodies suggested sexual transmission of *E. histolyrica* in the female population. The age range with the highest number of individuals positive for anti-*E. histolytica* antibodies was that of 25-29 years, with 11, and that of 30-

Table 2. Seroprevalence of anti-*Entamoeba histolytica* antibodies in the female outpatients from a gynecologist's office, Tokyo, Japan, by enzyme-linked immunosorbent assay 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-*Chlamydia trachomatis* (CT) antibodies that were also positive for anti-*E. histolytica* antibodies.

Table 3. Age distribution of the female outpatients from a gynecologist's office with positive for anti-*Entamoeba 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-*Chlamydia trachomatis* antibodies that were also positive for anti-*E. histolytica* antibodies are provided in parentheses.

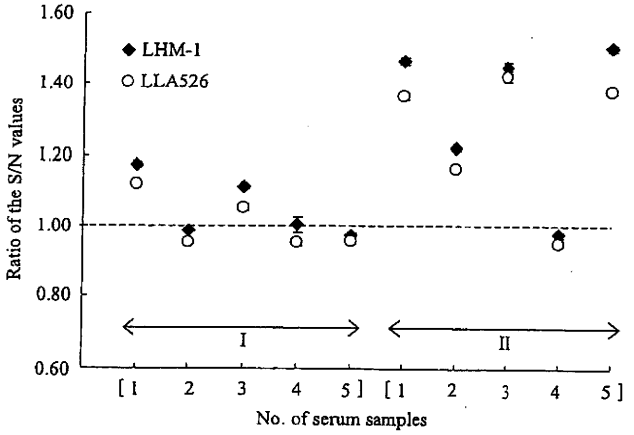

Fig. 1. Ratio of the S/N values of ELISA with better sensitivity performed using the antigens from hamster liver-passaged LHM-1 and LLA526 strains to the S/N values of ELISA using the antigen from the HM-1:IMSScl6 (HM-1) strain in the clinical serum samples of amoebiasis (S/N values: OD of serum sample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

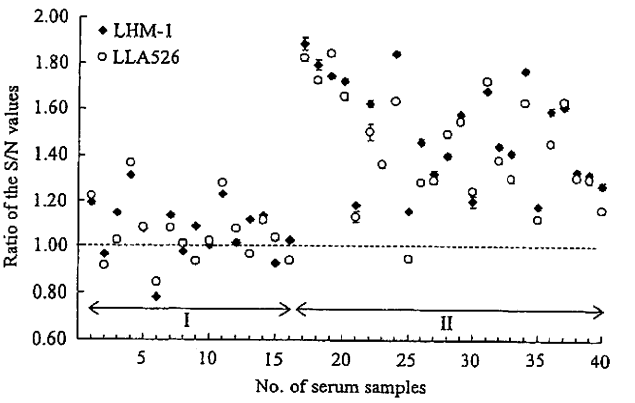

Fig. 2. Ratio of S/N values of ELISA performed using antigens from the LHM-1 and LLA526 strains to the S/N values of conventional ELISA performed using the antigen from the HM-1 strain in female outpatients from a gynecologist's office. The mean of the ratios of triplicate ELISA are plotted. I: Samples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

34 years, with 9. The number of individuals positive for anti-*C. tracomatis* antibodies correlated with the number of those positive for anti-*E. hitolytica* antibodies (Table 3).

Comparison of S/N values of ELISA with three different antigens: Figure 1 shows the results of a pilot study in which ELISA with LHM-1 and LLA526 antigens was conducted using sera from 5 human cases each of amoebic liver abscess and asymptomatic cyst passers. 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 only in asymptomatic cases with low S/N values (P < 0.05 by t test) and not in amoebic liver abscess 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 two 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 *E. histolytica* infection in the female population of Tokyo is increasing annually.

In addition, the result that 60% of the female study population who were anti-*E. histolytica* antibody-positive were also positive for anti-*C. tracomatis* antibodies, an indicator of STD, along with the diversity of sexual behavior suggested that a major proportion of females positive for anti-*E. histolytica* antibodies may have been infected with *E. histolytica* 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 case in the MSM population (11,12). We are currently conducting further epidemiological studies on the route of *E. histolytica* 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 of further evaluation, the improved ELISA is expected to be effective for detecting anti-*E. histolytica* antibodies from such asymptomatic patients who have low antibody titers. Moreover, the hamster liver-passaged *E. histolytica* may be applied as a sensitive antigen to other serodiagnostic methods, such as dot-ELISA (13) and immunofluorescence antibody tests (14).

Because of the public's indifference to STDs, the control of amoebiasis should start with efforts to raise public awareness of the risk of infection by sexual transmission Also simpler and more sensitive mass examination methods should be developed, such as the newly designed ELISA using the antigen extracted from the virulence-augmented *E. histolytica* strains, which have a better sensitivity for the diagnosis of amoebiasis.

ACKNOWLEDGMENTS

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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: 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 has previously been documented. In this study, a multiplex polymerase chain reaction (PCR) assay that could distinguish the JSK2004-type *E. histolytica*–like variant (JSK04-Eh-V) from *E. histolytica* and *Entamoeba dispar* using three newly designed primer sets for amplifying each specific DNA fragment from their small-subunit ribosomal RNA genes was developed and established. 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 six individuals of four species, including an Abyssinian colobus monkey, a De Brazza's guenon (including the individual from whom JSK2004 was isolated), a white-faced saki, and a Geoffroy's spider monkey. In addition, the autopsied individuals of an Abyssinian colobus and Geoffroy's spider monkey that died of amoebic liver abscess were also evaluated. DNA samples were also analyzed for specific genotypes based on the nucleotide sequencing of two 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 indicated that the *E. histolytica*–like variant infection in this zoo was caused by the same type (i.e., JSK04-Eh-V). An axenic culture medium (yeast extract–iron–maltose–dihydroxyacetone–serum) was developed based on the yeast extract–iron–gluconic acid–dihydroxyacetone–serum medium, which is designed for axenic culture of *E. dispar*. This new medium could be used for axenically culturing *E. histolytica*, JSK04-Eh-V, and *E. dispar* in a single medium.

Key words: *Entamoeba histolytica*–like variant, multiplex PCR, nonhuman primates, 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 two species: *E. histolytica* and *Entamoeba dispar* (nonpathogenic); earlier, this differentiation had been difficult to establish 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 polymerase chain reaction (PCR).[3,5]

In Japan, particularly during the last decade, *E. histolytica* infections have not been detected in nonhuman primates by PCR.[12,14] However, three recent articles reported three different pathogenic *E. histolytica*–like variants showing subtle variations in the small-subunit ribosomal RNA (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 of 98.47% with *E. dispar*. Previously it was 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 because of 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

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Table 1. Oligonucleotide primers used for polymerase chain reaction (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 GCT G	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

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, the identity of the JSK04-Eh-V strains in the zoo, determined by analyzing their polymorphic genotypes as a fingerprint for identifying the strain of *E. histolytica*, was investigated.

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

MATERIALS AND METHODS

Primates

In order to assess the prevalence of infection with JSK04-Eh-V, 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, were surveyed.[11] The primates, comprising 11 species, included three De Brazza's guenons (*Cercopithecus neglectus*), 11 Abyssinian colobus monkeys (*Colobus guereza*), two ring-tailed lemurs (*Lemur catta*), two mandrills (*Mandrillus sphinx*), one lesser slow loris (*Nycticebus pygmaeus*), two ruffed lemurs (*Varecia variegata*), one northern night monkey (*Aotus trivirgatus*), seven Geoffroy's spider monkeys (*Ateles geoffroyi*), 12 Japanese macaques (*Macaca fuscata*), five white-faced sakis (*Pithecia pithecia*), and one 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 to obtain three samples per individual. Prior to performing the 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 using an *E. histolytica*-specific antigen detection kit (*E. histolytica II* kit; TechLab, Blacksburg, Virginia 24060, USA). Tissue samples obtained from the primates that had liver abscesses were embedded in paraffin, stained with periodic acid–Schiff, and were examined for amoebae.

DNA preparation

Of the three stool specimens collected from each individual, the specimen that had the largest number of amoebic cysts was utilized for DNA preparation. The cysts were concentrated and partially purified using the modified formalin–ether sedimentation method, in which formalin was replaced with a phosphate-buffered solution (pH 7.4). Subsequently, the QIAamp® DNA stool mini-kit (Qiagen GmbH, Hilden 40724, Germany; catalog no. 51504) was used to isolate the genomic DNAs of amoebae. The genomic DNAs of the amoebae found in the two samples of abscesses obtained from the liver abscess of both the autopsied Abyssinian colobus monkey and Geoffroy's spider monkey that died of amoebic liver abscess; the two ref-

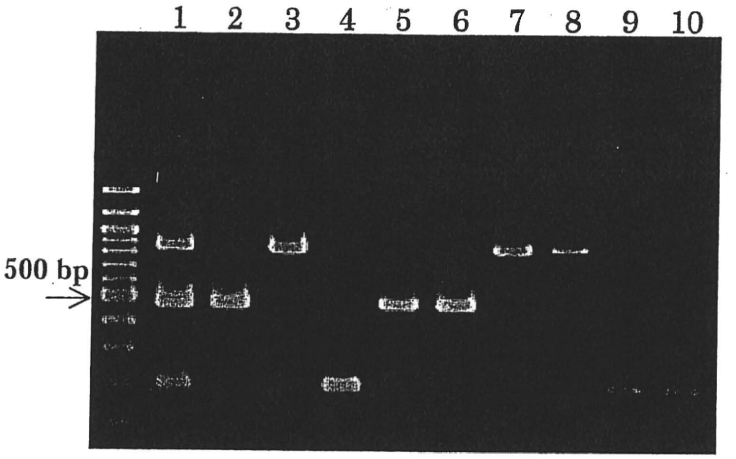

Figure 1. Polymerase chain reaction (PCR) products of the DNA samples from *Entamoeba histolytica*, JSK2004-type *E. histolytica*–like variant (JSK04-Eh-V), and *Entamoeba dispar* differentiated by multiplex PCR. Lane 1: mixture of the DNA templates of lanes 2, 3, and 4; Lane 2: DNA of HM-1:IMSScl6 (*E. histolytica*; length, 475 base pairs [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.

erence 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 (National Institutes of Health); and the JSK2004 clone 2 (JSK2004cl2; JSK04-Eh-V) were isolated using the QIAamp DNA mini-kit (Qiagen GmbH; catalog no. 51304).

Primers for multiplex PCR

The primers for multiplex PCR were designed based on the two 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 three 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 seven other intestinal parasitic protozoan and one nonprotozoan species: axenic trophozoites of *Entamoeba moshkovskii* (Laredo strain), *Entamoeba invadens* (IP-1 strain; ATCC no. 30994), and *Giardia intestinalis* (Portland-1 strain; ATCC no. 30888); cyst forms of *Escherichia coli* and *Cryptosporidium hominis*; culture form of *Blastocystis hominis* (nonprotozoan species) from human stool samples; and *E. coli*, *Entamoeba chattoni*, and *Entamoeba 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; catalog 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 sec, annealing for 40 sec 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 four cycles until it reached 56°C, after which the same temperature was maintained until the end of the cycling process.

Semi-nested PCR for SSU rRNA

In cases in which 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 ex*Taq*DNA polymerase (Takara Bio, Inc., Seta, Shiga 520-2134, Japan; catalog no. RR001A), 0.4 μM of each primer, and 0.25 mM of deoxynucleoside triphosphate. The follow-

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 polymerase chain reaction (PCR) and microscopic examination in a zoo of Tokyo, Japan.

Primate species	Common name	No. of samples	Positive number by multiplex PCR (symptoms)			Antigen detection[a]	Microscopic examination[b]
			Entamoeba histolytica	JSK04-Eh-V	*Entamoeba 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	

[a] *E. histolytica*-specific antigen detection kit [*E. histolytica II* kit (TechLab)] was used for examination of stool samples.

[b] *E. coli*: *Entamoeba coli*; *E. nana*: *Endolimax nana*; *G. intestinalis*: *Giardia intestinalis*; *E. chattoni*: *Entamoeba chattoni*; *C. mesnili*: *Chilomastix mesnili*. *E. chattoni* and *E. hartmanni* were identified by PCR. The numbers within the parentheses represent the number of positive cases..

[c] ALA, amebic liver abscess (death).

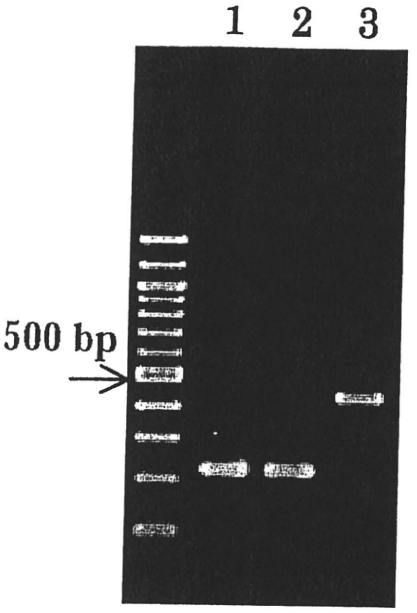

Figure 2. Differentiated polymerase chain reaction (PCR) products of the DNA samples of *Entamoeba chattoni* and *Entamoeba hartmanni* using PCRs. Lane 1: The product (length, 215 base pairs [bp]) from an *E. chattoni* DNA sample (stool) from a mandrill; Lane 2: The product (length, 215 bp) from an *E. 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.

ing cycling parameters were utilized: *Taq* activation at 94°C for 3 min; 35 cycles of denaturation at 94°C for 40 sec, annealing at 58°C for 40 sec, and extension at 72°C for 1 min; and extension at 72°C for 5 min.

PCR for *E. chattoni* and *E. hartmanni*

Entamoeba chattoni and *E. hartmanni* were identified by PCR assays using two 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 LA*Taq*DNA polymerase, 0.4 μM of each primer, and 0.25 mM of deoxynucleoside triphosphate. The following cycling parameters were utilized: *Taq* activation at 94°C for 3 min; 35 cycles of denaturation at 94°C for 40 sec, annealing at 55°C for 40 sec, and extension at 72°C for 1 min; and extension at 72°C for 5 min.

Polymorphic gene analysis

The genotyping of JSK04-Eh-V was reexamined to determine whether it would have the same genotype as JSK2004cl2. This was performed based on the nucleotide sequences of two protein-coding (chitinase and SREHP) genes and the protein-non-coding 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 LA*Taq*DNA polymerase (Takara Bio, Inc.; catalog no. RR02AG), 0.4 μM of each primer, and 0.25 mM of deoxynucleoside triphosphate. The following cycling parameters were utilized: *Taq* activation at 94°C for 3 min; 35 cycles of denaturation at 94°C for 40 sec, annealing at 50°C (chitinase, SREHP, and locus 1-2) or 56°C (SSU rRNA) for 40 sec, and extension at 72°C for 1 min; and extension at 72°C for 5 min.

Sequence analysis

Sequence analysis was performed. 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 using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California 94404, USA; catalog no. 4337455) and an ABI PRISM 3100 Genetic Analyzer.

Yeast extract–iron–maltose–dihydroxyacetone–serum (YIMDHA-S) medium

An axenic culture medium, namely, YIMDHA-S, based on the yeast extract–iron–gluconic acid–dihydroxyacetone–serum medium (YIGADHA-S)[6] designed for the axenic culture of *E. dispar*, was designed for the isolation and culture of *E. histolytica*, the *E. histolytica*–like variant, and *E. dispar* in a single medium. YIGADHA-S differs from the existing YIMDHA-S medium in that gluconic acid, 0.5% in YIGADHA-S, is replaced with an equal concentration of maltose in YIMDHA-S. 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, the effectiveness of several commercially available yeast extracts purchased from different manufacturers as an ingredient of YIMDHA-S was evaluated. Except for the standard stock obtained from BBL (Becton Dickinson Co., Cockeysville, Maryland 21030, USA; catalog no. 4311929; lot no. 1000I9DHJT), among all the yeast extracts test-

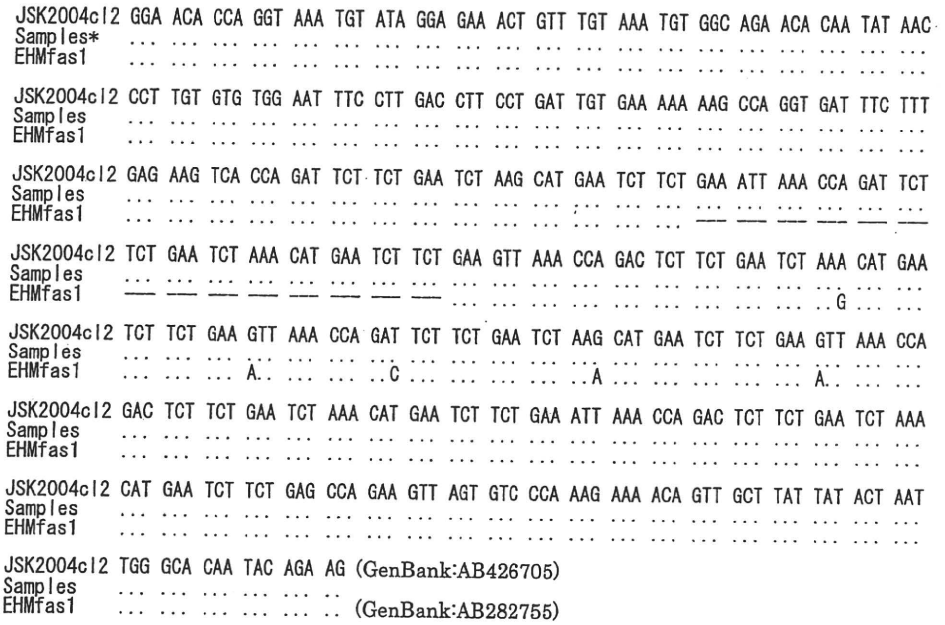

Figure 3. Comparison of the chitinase sequences of the *Entamoeba histolytica*–like variant strain JSK2004cl2 and *E. histolytica*–like variants detected from *Ateles geoffroyi* and *Pithecia pithecia* with the sequences of the reference *E. histolytica*–like variant strain isolated from *Macaca fascicularis*. 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 (one pus sample from a liver abscess) and white-faced saki (two 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]

ed, only that obtained from Merck (Merck KGaA, Damstadt 64271, Germany; catalog 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 base pairs (bp), 848 bp, and 195 bp, respectively (Fig. 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* (Fig. 1). The specificity of multiplex PCR was examined by analyzing the templates of the DNA extracted from seven other intestinal parasitic protozoan and one nonprotozoan species, as previously described. None of the PCR products was observed on multiplex PCR examination of these parasites.

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 trophozoites 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.

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JSK2004cl2 AAG AAA AAG AAA AAA GTA GCT CAG CAA AAC CAG AAT CAA GTT CAA ACA AAG ATA ATG AAG
EHMfas1     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
P19         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

JSK2004cl2 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. ... ... ... ... ... ... ... ... ... ...

JSK2004cl2 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.

JSK2004cl2 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.. ... ...

JSK2004cl2 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 ... ... ... ... ... ... ... ... ... ... ... ...

JSK2004cl2 CAG AAG CAA GTT CAA GTG ATA AAC CAG ATA ACA AAC CAG AAG CAA GTT CAA GTG ATA AAC
EHMfas1     ... ... ... .C. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
P19         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .C. ... ... ... ...

JSK2004cl2 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.

JSK2004cl2 CAA CTA ATA AAC CAG AAG CAA GTT CAA CTA ATA AAC CAG AAG CAA GTT CAA CTA GTA ATT
EHMfas1     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
P19         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

JSK2004cl2 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. Comparison of the sequences in the SREHP of the *Entamoeba 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]

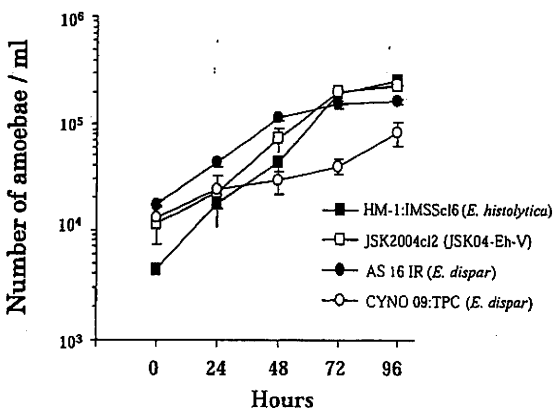

Figure 5. The growth kinetics of the axenically cultured HM-1:IMSScl6, JSK2004cl2, AS 16 IR, and CYNO 09:TPC strains in the yeast extract–iron–maltose–dihydroxyacetone–serum (YIMDHA-S) medium. The mean numbers of amoebae in duplicate cultures have been plotted.

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 six individuals of four primate species: Abyssinian colobus monkey; De Brazza's guenon, including the individual from which JSK2004 was isolated; white-faced saki; and Geoffroy's spider monkey. In addition, the autopsied samples of the Abyssinian colobus monkey and Geoffroy's spider monkey that died of amoebic liver abscess were positive for JSK04-Eh-V. 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 six primates were subjected to PCR to detect the fragments of the chitinase, SREHP, and locus 1-2 genes. The PCR products of the chitinase genes from the DNA samples of two primates and JSK2004cl2 and the PCR products of the locus 1-2 genes from the DNA samples of four primates and JSK2004cl2 were sequenced; however, the PCR products of the SREHP genes were obtained only from the DNA sample of JSK2004cl2. There was perfect homology between the sequences of the PCR products of the chitinase genes obtained from two primates (one Geoffroy's spider monkey and one white-faced saki) and the PCR products of the locus 1-2 genes from four primates (two Abyssinian colobus monkeys, one Geoffroy's spider monkey, and one white-faced saki). Moreover, the sequences of the two genes of JSK2004cl2 (locus 1-2: GenBank accession no. AB426704; chitinase: GenBank accession no. AB426705) also demonstrated perfect homology. However, the sequence data of the chitinase and SREHP genes of the other two types of *E. histolytica*–like variants (GenBank accession nos. AB282755 and AB197935) isolated from the cynomolgus and rhesus monkeys were different compared to the sequence data of the chitinase gene and SREHP gene (GenBank accession no. AB426706) of JSK2004cl2 (Figs. 3, 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 three 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 the zookeepers who were 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 third 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 three 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 five primates, except for JSK2004cl2, could not be amplified by PCR, the polymorphic chitinase and locus 1-2 gene sequences from three and six primates, respectively, were observed to be identical. The reasons for the inability of PCR to amplify the SREHP gene were thought to be related to the small amount of JSK04-Eh-V DNA in the stool and liver abscess samples, which were insufficient for the PCR, and the presence of a few irrelevant PCR fragments in each case. Therefore, JSK04-Eh-V infections that oc-