Isolation and characterization of a potentially virulent species *Entamoeba nuttalli* from captive Japanese macaques

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SUMMARY

We have recently proposed revival of the name *Entamoeba nuttalli* Castellani, 1908 for a virulent amoeba (P19-061405 strain) isolated from a rhesus monkey (*Macaca mulatta*) and located phylogenetically between *E. histolytica* and *E. dispar*. In this study, *E. nuttalli* was isolated from feces of captive Japanese macaques (*M. fuscata*) in an open-air corral in Japan. The sequence of the 18S rRNA gene in the isolates differed from the P19-061405 strain in 2 nucleotide positions, but was identical to the EHMfas1 strain isolated previously from a cynomolgus monkey (*M. fascicularis*). One of the *E. nuttalli* isolates from Japanese macaques, named the NASA6 strain, was axenized and cloned. In isoenzyme analysis, the mobilities of hexokinase and phosphate glucose isomerase in the NASA6 strain were identical to those in the P19-061405 and EHMfas1 strains, but the mobility of phosphoglucomutase was different. These results were supported by gene analyses of these enzymes. Inoculation of NASA6 strain trophozoites into the liver of hamsters led to formation of an amoebic liver abscess. The liver lesions were characterized by extensive necrosis associated with inflammatory reactions. These results demonstrate that the NASA6 strain is potentially virulent and that *E. nuttalli* should be recognized as a common parasite in macaques.

Key words: *Entamoeba nuttalli*, Japanese macaque, 18S rRNA gene, virulency, zymodeme.

INTRODUCTION

The protozoan parasite *Entamoeba histolytica* is responsible for millions of cases of amoebic colitis and liver abscess in humans annually, resulting in up to 100 000 deaths (World Health Organization, 1997). Another intestinal-dwelling amoeba, *E. dispar*, is morphologically indistinguishable from *E. histolytica* but is non-pathogenic (Diamond and Clark, 1993). *E. histolytica*/*E. dispar* is also commonly found in the feces of non-human primates (Smith and Meerovitch, 1985; Muriuki *et al*. 1998; Tachibana *et al*. 2000; Verweij *et al*. 2003). *E. histolytica* infection in these primates is a serious problem for animal health and also has zoonotic potential, which makes it important to discriminate *E. histolytica* from other non-pathogenic amoebae.

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In older literature, several *E. histolytica*-like amoebae in monkeys have been described. The name *E. nuttalli* Castellani, 1908 was proposed for an amoeba found in a liver abscess in a *Macacus pileatus* monkey in Colombo, Sri Lanka (Castellani, 1908). Thereafter, *E.* (*Löschia*) *duboscqi* Mathis, 1913; *E. chattoni* Swellengrebel, 1914; *E. cercopitheci* Macfie, 1918; and *E. ateles* Suldey, 1924 have been described as *E. histolytica*-like species from non-human primates (Wenyon, 1965). However, except for *E. chattoni*, which has uninucleated cysts, these species are thought to be synonymous with *E. histolytica* (Hegner and Schumaker, 1928; Neal, 1966).

We have recently isolated an *E. histolytica*-like amoeba (P19-061405 strain) from a rhesus monkey (*Macaca mulatta*) in Nepal (Tachibana *et al*. 2007). However, the rRNA gene sequence of the isolate is located phylogenetically between *E. histolytica* and *E. dispar*. Furthermore, inoculation of trophozoites of the P19-061405 strain into livers of hamsters causes amoebic liver abscesses, indicating that the strain is potentially virulent. Therefore, we have proposed the revival of the name *E. nuttalli* for the

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amoeba isolated from rhesus monkey. Two other *E. histolytica*-like amoebae, the EHMfas1 and JSK2004 strains, which are also located phylogenetically between *E. histolytica* and *E. dispar*, have been isolated from a cynomolgus monkey (*M. fascicularis*) and a De Brazza's guenon (*Cercopithecus neglectus*), respectively, although there are 1–3 nucleotide differences among the 18S-rRNA genes in these 3 isolates (Suzuki *et al*. 2007; Takano *et al*. 2007).

Japanese macaque (*M. fuscata*) is a monkey that is distributed widely in Japan. A high prevalence of *E. dispar* infection but not of *E. histolytica* has been found in this primate (Rivera and Kanbara, 1999; Tachibana *et al*. 2001), which makes it of interest to examine whether *E. nuttalli* infections are found in Japanese macaques. In this study, we report a high prevalence of *E. nuttalli* infection in captive Japanese macaques, and we describe the characteristics of the amoeba after isolation in an axenic culture.

MATERIALS AND METHODS

Subjects and stool examination

Thirty stool samples from captive Japanese macaques were collected in an open-air corral located in a park in Nagasaki City, Japan, between December 2006 and March 2008. The samples were examined microscopically with iodine staining. Aliquots of each sample were suspended in 2% potassium dichromate solution and stored at room temperature.

Culture conditions

Fresh stool samples (within 6 h after collection) were suspended in water for 24 h and then cultured in modified Tanabe-Chiba medium at 37 °C (Tachibana *et al*. 2007). Grown trophozoites were transferred to Robinson's medium (Robinson, 1968). After several passages, the trophozoites were treated with a cocktail of antibiotics and then cultured monoxenically with living *Crithidia fasciculata* in TYI-S-33 medium (Diamond *et al*. 1978) supplemented with 15% adult bovine serum at 37 °C. Finally, the trophozoites were cultured axenically in TYI-S-33 medium and then cloned by limiting dilution, followed by microscopic observation. Trophozoites of the *E. nuttalli* P19-061405 strain and the *E. histolytica* SAW1453 and HM-1:IMSS strains were also cultured axenically in TYI-S-33 medium. Trophozoites of *E. dispar* SAW1734RclAR were cultured monoxenically with autoclaved *C. fasciculata* in YIGADHA-S medium supplemented with 15% adult bovine serum at 37 °C (Kobayashi *et al*. 2005).

Sandwich ELISA

Fresh stool samples (within 6 h after collection) were processed using an *E. histolytica* II kit (TechLab) for detection of *E. histolytica* antigen. Approximately 10^4 trophozoites cultured axenically were also analysed using the same kit.

Determination of the trophozoite diameter

Diameters of trophozoites were measured using a method reported in the literature (López-Revilla and Gómez-Domínguez, 1988). Briefly, cultured trophozoites in log growth phase were chilled on ice and then fixed with 2·5% glutaraldehyde in 0·1 M cacodylate buffer (pH 7·4). After washing with phosphate-buffered saline (PBS), the diameters of 100 round trophozoites were determined with an ocular micrometer. Statistical analysis was performed by Student's *t*-test.

Isolation of genomic DNA

Small aliquots of fecal samples in potassium dichromate solution were mixed with ether and centrifuged. Precipitates were washed 3 times with PBS and genomic DNA was then isolated using a DNeasy tissue kit (Qiagen). Genomic DNA was also isolated from cultured trophozoites using the same kit.

PCR amplification

For detection of various *Entamoeba* species, genomic DNA was subjected to 35 cycles of PCR amplification using Takara *ExTaq* DNA polymerase. Partial 18S rRNA genes of *E. histolytica*, *E. dispar* and *E. nuttalli* were amplified using primer sets described previously (Tachibana *et al*. 2007). Amplification of the partial 18S rRNA gene of *E. coli* was performed using the primers 5′-GAA TGT CAA AGC TAA TAC TTG ACG-3′ and 5′-GAT TTC TAC AAT TCT CTT GGC ATA-3′, which were designed based on sequences in the GenBank database (Accession numbers: AF149914 and AF149915). Detection of the *E. chattoni* partial 18S rRNA gene was performed using primers Echattoni1 and Echattoni2 (Verweij *et al*. 2001). PCR conditions were as follows: denaturation at 94 °C for 15 s (195 s in cycle 1), annealing at 55 °C (for *E. chattoni*) or 60 °C (for others) for 30 s, and extension at 72 °C for 30 s (450 s in cycle 35). An approximately 2·4-kb region containing the 18S and 5·8S rRNA genes was amplified from genomic DNA of cultured trophozoites over 30 cycles using Takara *ExTaq* DNA polymerase (Tachibana *et al*. 2007), using the following PCR conditions: denaturation at 94 °C for 15 s (195 s in cycle 1), annealing at 60 °C for 30 s, and extension at 72 °C for 180 s (600 s in cycle 30). Amplification of a serine-rich protein gene was performed essentially as reported by Ghosh *et al*. (2000). The genes encoding hexokinase (HXK) and glucose phosphate isomerase (GPI) were amplified in 30 cycles using PrimeSTAR HS DNA polymerase

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(Takara) with primers described previously (Tachibana *et al*. 2007). The phosphoglucomutase (PGM) gene was amplified using the primers 5′-TCG TTG AAC CAG ATC AGT GC-3′ and 5′-AAG CTT CTC TGG ATG GTG TTG-3′, as described in Takano *et al*. (2009). The PCR conditions using PrimeSTAR HS DNA polymerase were as follows: denaturation at 98 °C for 10 s, annealing at 55 °C (for *HXK* and *GPI*) or 60 °C (for *PGM*) for 5 s, and extension at 72 °C for 90 s.

Sequencing

PCR products of rRNA genes, serine-rich protein genes, and *PGM* genes were subjected to direct sequencing after purification using a QIAquick PCR purification kit (Qiagen). PCR products of the *HXK* and *GPI* genes were processed with a Zero Blunt TOPO PCR cloning kit for sequencing (Invitrogen). Six clones of each gene were sequenced using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). The reactions were run on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Zymodeme analysis

Isoenzyme analysis of cultured trophozoites in starch gel was performed using a literature method (Sargeaunt, 1988), with determination of the mobilities of 4 enzymes: HXK, GPI, PGM, and L-malate:$NADP^+$ oxidoreductase (ME).

Hepatic inoculation

Five male Syrian hamsters (*Mesocricetus auratus*) weighing 95–110 g were purchased from Japan SLC, Inc. The hamsters were anaesthetized by intraperitoneal injection of pentobarbital and then 5×10^5 trophozoites cultured axenically were inoculated into the left lobe of the liver. The hamsters were sacrificed 7 days after inoculation and formation of amoebic liver abscesses was examined. Representative liver samples including abscesses were fixed with 4% paraformaldehyde in phosphate buffer and embedded in paraffin. Sections were stained with haematoxylin and eosin (H&E) and PAS (periodic acid-Schiff), and examined by standard light microscopy (Olympus BX51).

Electron microscopy

Small pieces of liver close to an abscess were fixed in periodate/lysine/ paraformaldehyde (PLP) (McLean and Nakane, 1974) overnight at room temperature. The samples were washed with 0·1 M phosphate buffer (pH 7·4), post-fixed with 1% OsO_4 in 50 mM phosphate buffer for 1 h at 4 °C, and then dehydrated with a graded ethanol series and embedded in Quetol 812 (Nisshin EM). Ultrathin sections were stained with uranyl acetate and lead citrate and then examined using a JEOL JEM-1200 EX II transmission electron microscope.

RESULTS

Detection of amoebae by stool examination and PCR

The thirty stool samples were all formed, and not loose, watery or bloody. Cysts of *E. histolytica*/*E. dispar*/*E. nuttalli*, *E. chattoni* and *E. coli* were highly prevalent in microscopic observation. PCR amplification using primers specific for *E. nuttalli* yielded products from all samples, whereas no samples gave products using primers for *E. histolytica* and *E. dispar*. *E. chattoni* and *E. coli* were detected by PCR in all samples and the amplicons were confirmed to be from the expected species by sequencing. Examination of the 30 fecal samples using an *E. histolytica* antigen detection kit gave no positive results.

Isolation of E. nuttalli *in culture*

Growth of trophozoites in Tanabe-Chiba medium was observed in 4 samples. After several passages, *E. nuttalli* DNA was detected by PCR in these samples, but *E. chattoni* and *E. coli* DNA was not amplified. Three of the 4 samples were used as xenic strains and labelled as NASA821, NASA823 and NASA829 for subsequent experiments. One isolate, named the NASA6 strain, was axenized and then cloned. The living trophozoites in the *E. nuttalli* NASA6 strain had an elongated shape of length 18–60 μm. The average diameter of chilled trophozoites of the NASA6 strain ($22{\cdot}4 \pm 0{\cdot}43$ (mean ± S.E.) μm) was significantly smaller than that of the *E. histolytica* HM-1:IMSS strain ($28{\cdot}3 \pm 0{\cdot}54\,\mu$m, $P < 0{\cdot}0001$), but larger than that of the *E. nuttalli* P19-061405 strain ($20{\cdot}7 \pm 0{\cdot}29\,\mu$m, $P = 0{\cdot}001$). Trophozoites of the NASA6 strain cultured axenically gave a positive result with an *E. histolytica* II antigen detection kit.

Analysis of ribosomal RNA genes in the isolates

The 18S rRNA gene of a clone of the NASA6 strain (DDBJ, EMBL, and GenBank Accession number AB485592) showed 2 nucleotide differences compared with the P19-061405 strain (AB282657), but was identical to the EHMfas1 strain (AB197936). There were no differences in the 5·8S rRNA gene and the internal transcribed spacer (ITS) 1 and 2 regions among the 3 strains. The sequences of the rRNA genes from the 3 xenic isolates were also identical to that of the NASA6 strain.

Analysis of the serine-rich protein gene

The deduced amino acid sequence from the serine-rich protein gene of the NASA6 strain

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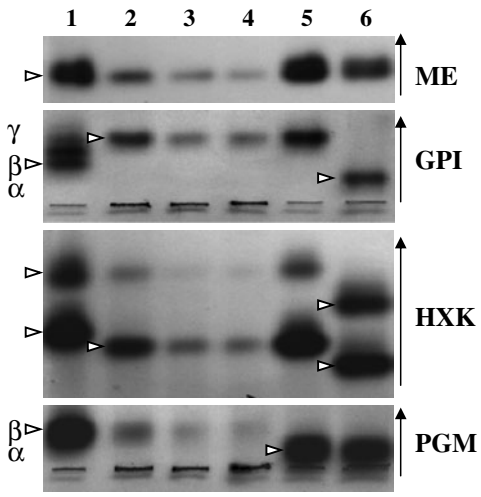

Fig. 1. Isoenzyme patterns for ME, GPI, HXK and PGM of *Entamoeba nuttalli* strains obtained from Japanese macaques (lanes 2 to 4). Lane 1, *E. histolytica* SAW1453 (Zymodeme XIV); lane 2, *E. nuttalli* NASA6 (axenic); lane 3, *E. nuttalli* NASA821; lane 4, *E. nuttalli* NASA829; lane 5, *E. nuttalli* P19-061405; lane 6, *E. dispar* SAW1734RclAR (Zymodeme I). Vertical arrows indicate the direction of migration. Arrowheads indicate positions of bands.

(AB485593) differed from those of the P19-061405 (AB282662) and EHMfas1 (AB197935) strains. In the NASA6 strain, the amino acid motifs EKASSSDKP(cca), EASSN(aat)DKP and EASSSDKS(tca) were present instead of EKAS-SSDKS(tca), EASSS(agt)DKP and EASSSDKP (cca), respectively, in the P19-061405 and EHMfas1 strains, resulting in single nucleotide substitutions. In addition, the ESSSN(aat)DKP motif was found in the NASA6 strain, for which ESSSS(agt)DKP is the closest sequence motif in human isolates. The sequence in the region with glutamic and aspartic acids in the NASA6 strain was similar to that in EHMfas1, but insertion of an additional ED was found in NASA6. The sequences of serine-rich protein genes in the 3 xenic strains were identical with that of the NASA6 strain, suggesting that only 1 type of *E. nuttalli* was prevalent in the monkeys in the corral.

Analyses of isoenzyme patterns

The electrophoretic patterns of 4 enzymes from the axenic strain (NASA6) and xenic strains (NASA821 and NASA829) of *E. nuttalli* isolated from Japanese macaques are shown in Fig. 1. The patterns for HXK and GPI were identical to those observed in the *E. nuttalli* P19-061405 strain and differed from those of *E. histolytica* and *E. dispar*. However, the mobility of the PGM band in the 3 NASA strains was identical with that in *E. histolytica*, whereas the mobility of this band in the P19-061405 strain was identical with that in *E. dispar*.

Analyses of HXK, GPI *and* PGM *genes*

The calculated molecular mass and theoretical p*I* of HXK1 (AB485594) in the *E. nuttalli* NASA6 strain were 49·8 kDa and 5·38, respectively, and those for HXK2 (AB485595) were 49·3 kDa and 4·99, respectively, with both enzymes containing 445 amino acids. These p*I* values were identical to those for the *E. nuttalli* P19-061405 strain (AB282663 and AB282664), although HXK1 and HXK2 differed by 1 and 2 amino acids, respectively: Ala^{75} in both enzymes in NASA6 compared to Pro^{75} in P19-061405, and Ile^{232} in HXK2 in NASA6 compared to Val^{232} in P19-061405. The amino acid sequences of GPI1 (AB485596) and GPI2 (AB485597) in the *E. nuttalli* NASA6 strain were consistent with the calculated molecular mass of 61·4 kDa and theoretical p*I* of 6·6. These values were also identical to those of the P19-061405 strain (AB282665 and AB282666). Each protein had a single amino acid change from NASA6 to P19-061405: Ile^{232} to Val^{232} in GPI1, and Ala^{330} to Val^{330} in GPI2. *PGM* genes from *E. nuttalli* NASA6 and P19-061405 strains (AB485598 and AB485599) encoded proteins of 553 amino acids with calculated molecular masses of 60·8 kDa. Amino acid differences were detected in 2 positions: Asn^{416} and Asp^{441} in NASA6 compared to Ser^{416} and Asn^{441} in P19-061405. There were 4 differences in amino acids between NASA6 and *E. histolytica* (CAA74796), and 11 differences between NASA6 and *E. dispar* (CAA74797). The p*I* values of PGM from the NASA6 and P19-061405 strains, 5·87 and 5·99, were identical to those from *E. histolytica* and *E. dispar*, respectively.

Virulence in hamsters

Of the 5 hamsters that received intrahepatic inoculation of trophozoites of the NASA6 strain, 1 died at 6 days after inoculation and another died at 7 days after inoculation. In these hamsters, the abscess weights as a percentage of the whole liver were 30% and 35%, respectively, and trophozoites were found in the abscesses in both animals. The other 3 hamsters were sacrificed at 7 days after inoculation and amoebic liver abscesses were also found in these animals. Histopathological analysis of the liver damage produced by *E. nuttalli* trophozoites showed large and irregular necrotic areas of liver parenchyma. These areas were peripherally limited by abundant live and pleomorphic trophozoites associated mainly with chronic inflammatory infiltrates (Fig. 2), forming palisade bands of epithelioid cells with evidence of a granulomatous reaction. The necrotic

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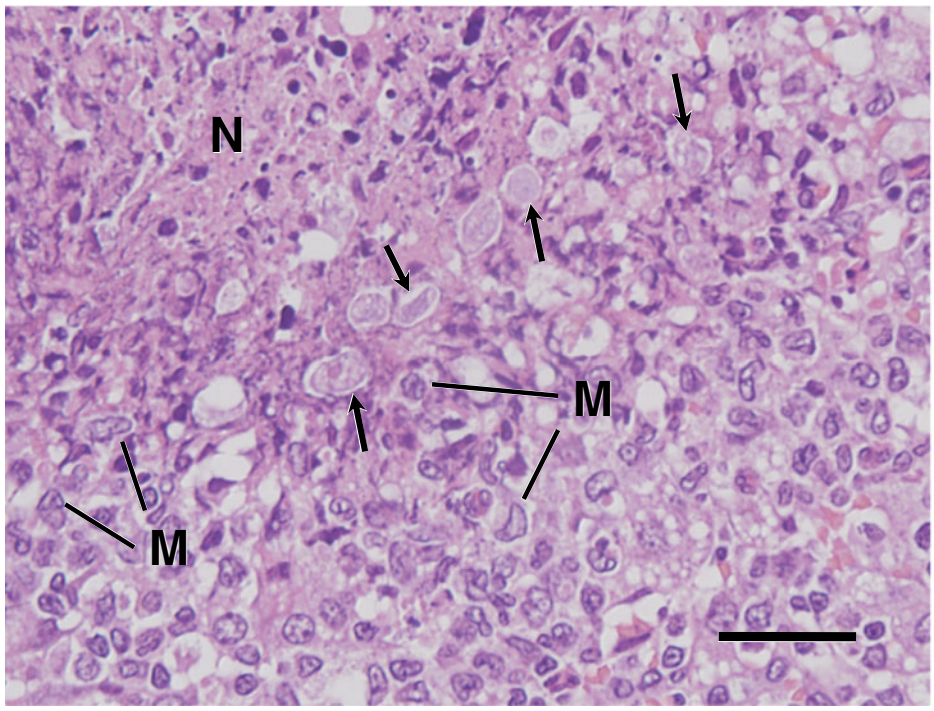

Fig. 2. Liver histology in hamsters inoculated with 5×10^5 trophozoites of the *Entamoeba nuttalli* NASA6 strain. At 7 days after inoculation, areas of necrosis (N) appeared that were limited peripherally by multiple trophozoites (arrows) and mononuclear (M) inflammatory cells. (H&E stain, Scale bar = 40 μm).

zones contained homogeneous and eosinophilic material with cell remnants and dense basophilic deposits.

Electron microscopy of hamster liver inoculated with E. nuttalli *trophozoites*

Electron microscopy of hepatic tissues taken from the vicinity of abscess walls at 7 days after inoculation showed the presence of elongated and pleomorphic trophozoites of *E. nuttalli* that were randomly spread at the outer limits of the abscess. The parasites were surrounded by abundant necrotic tissue formed by hepatic and inflammatory cell remnants composed of small vesicles and dense granules, membrane fragments and damaged cells (Fig. 3A). Some of these cells were partially damaged hepatocytes with cytoplasm containing multiple vesicles, lipid droplets and dilated organelles. Other damaged cells that were closely associated with the parasites and compatible with lysed inflammatory cells are shown in Fig. 3B. These irregularly outlined damaged cells surrounding the trophozoite contained only a fine granular material with a few dense granules distributed mainly at the periphery. Some trophozoites showed initial endocytic activity of damaged cells characterized by concavity at one pole of the parasite associated with an unrecognized damaged cell (Fig. 3B). The nuclei of the trophozoites were round but slightly irregular, and sometimes had an undulating profile (Fig. 3A). Dense chromatin was distributed at the periphery close to the nuclear membrane. The plasma membrane of each trophozoite was intact, thin and sharply defined. The cytoplasm included multiple phagocytic vacuoles of different sizes, with some containing membranous, amorphous or microvesicular material or other cell remnants (Fig. 3A and B). Geometrically arranged crystalloid structures were occasionally observed and glycogen particles were scarce.

DISCUSSION

This study is the first report of isolation of *E. nuttalli* from Japanese macaques. Since *E. nuttalli* was detected in all fecal samples, Japanese macaques appear to be highly susceptible to *E. nuttalli* infection, as well as to *E. coli* and *E. chattoni*. Surprisingly, *E. dispar* was not detected, whereas our previous studies have demonstrated that *E. dispar* is prevalent in Japanese macaques (Rivera and Kanbara, 1999; Tachibana *et al.* 2001). This suggests that the amoeba species prevalent in captive macaques may vary in each colony and may be different from that of wild macaques. The Japanese macaque (*M. fuscata*) is phylogenetically closer to rhesus monkey (*M. mulatta*) than cynomolgus monkey (*M. fascicularis*) (Hayasaka *et al.* 1996; Chu *et al.* 2007), but the sequence of the 18S rRNA gene of the NASA6 strain differed from that of the P19-061405 strain isolated from a rhesus monkey but was identical to that of the EHMfas1 strain isolated from a cynomolgus

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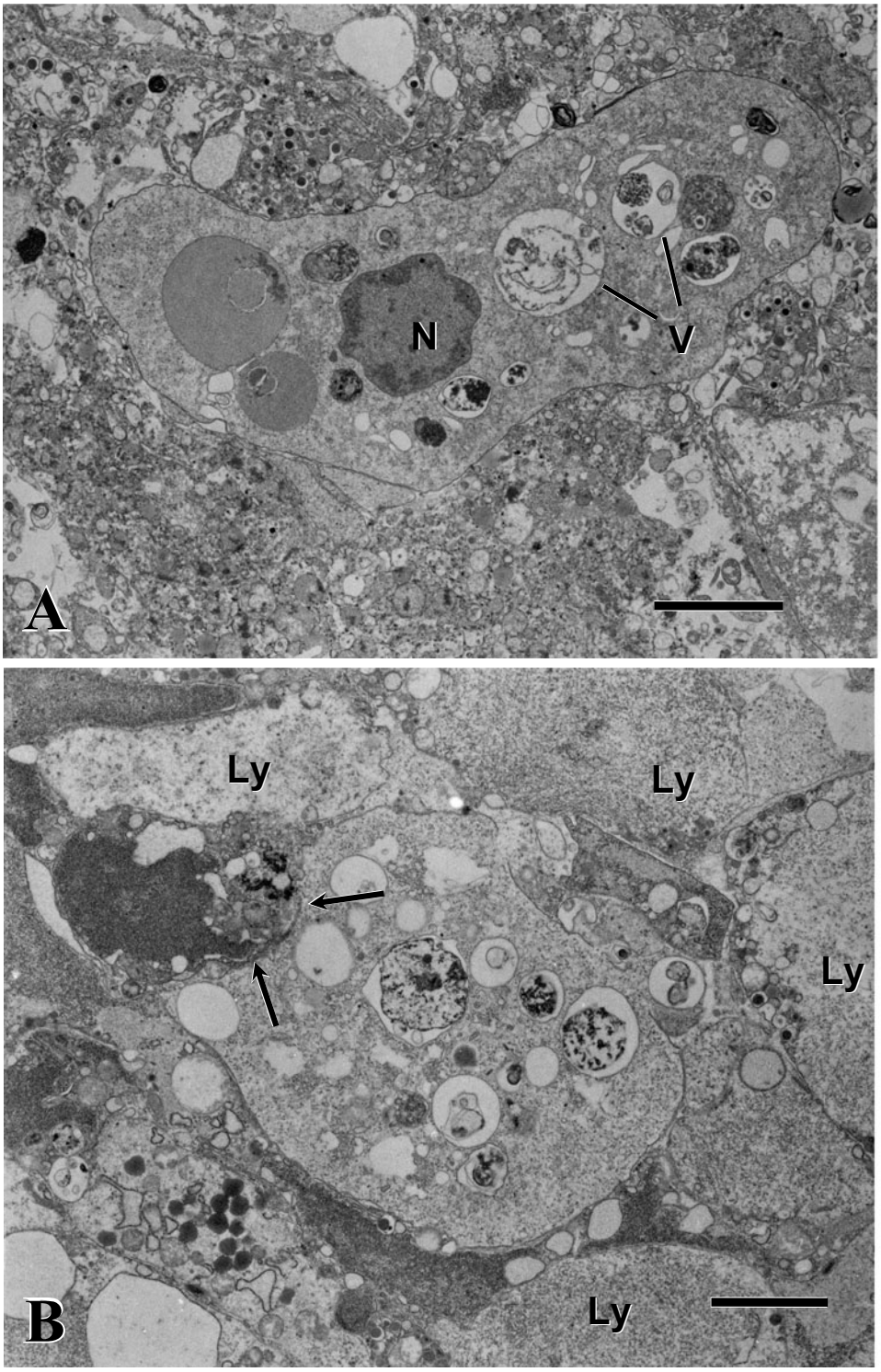

Fig. 3. Transmission electron micrographs of hamster liver at 7 days after inoculation of *Entamoeba nuttalli* NASA6 trophozoites. (A) The image shows an irregularly elongated trophozoite with a round nucleus (N) with peripherally distributed chromatin and a cytoplasm containing multiple vacuoles (V) enclosing membranous or amorphous material and granules of unknown origin. The thin, sharp and intact plasma membrane of the amoeba is apparent. The parasite is surrounded by abundant damaged host cells. (B) The image shows an ovoid trophozoite of *E. nuttalli* in the initial stage of the endocytic process (arrows) with a damaged cell contacting the left pole of the cell. The cytoplasm of the parasite contains vacuoles of different sizes containing unknown material. Multiple lysed (Ly) cells containing a fine granular material with small dense granules are seen surrounding the amoeba. (Scale bar = 3 μm).

monkey. At present, the number of *E. nuttalli* isolates is insufficient to establish the relationship of evolution and dispersal in hosts and parasites, but the NASA6 and EHMfas1 strains clearly differ based on the serine-rich protein gene sequences and isoenzyme patterns.

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In zymodeme analysis, the location of the slower running band for HXK and the appearance of a single γ band for GPI may be unique characteristics of *E. nuttalli*-type amoebae, compared with *E. histolytica* and *E. dispar*. In contrast, the pattern for PGM was distinctly different in NASA strains compared with other *E. nuttalli*-type strains in zymodeme analysis, with a β band in the NASA strains and an α band in the other strains. Sargeaunt has suggested that the absence of an α band and the presence of a β band for PGM are characteristics of *E. histolytica* that distinguish it from *E. dispar*, with the exception of zymodeme XIII (Sargeaunt, 1988). NASA6 was the first strain showing *E. histolytica*-type mobility for PGM, while the P19-061405, EHMfas1 and JSK2004 strains showed *E. dispar*-type mobility (Tachibana *et al*. 2007; Takano *et al*. 2007; Suzuki *et al*. 2007). However, gene analyses have shown that there are fewer differences in the amino acid sequence of PGM between the *E. nuttalli* NASA6 and P19-061405 strains than between NASA6 and *E. histolytica* or P19-061405 and *E. dispar*.

Cultured trophozoites of the NASA6 strain were scored positive by the *E. histolytica* antigen detection kit, similar to the P19-061405 and EHMfas1 strains (Takano *et al*. 2005; Tachibana *et al*. 2007). However, none of the 30 fecal samples were found to be positive using this kit. Suzuki *et al*. (2007) have reported positive results in fecal samples analysed with this kit, but the samples were tested after a freezing-thawing procedure (personal communication). Therefore, the amount of antigen extracted may account for the different results. It is also possible that the anti-*E. histolytica* lectin antibody in the kit has low reactivity to *E. nuttalli* and comparative analysis of the antigen is required to address this issue.

Morphological differences of *E. nuttalli* compared with *E. histolytica* and *E. dispar* were not evident in cysts and xenically cultured trophozoites under light microscopy. However, trophozoites of *E. nuttalli*, as well as those of *E. histolytica*, were axenized easily in comparison with *E. dispar* (Clark, 1995; Kobayashi *et al*. 2005). The length range of living trophozoites of *E. nuttalli* in axenic culture overlaps with that of *E. histolytica*, but the *E. nuttalli* trophozoites tend to be elongated in comparison with *E. histolytica* trophozoites (Espinosa-Cantellano *et al*. 1998). The average diameters of chilled trophozoites of *E. nuttalli* NASA6 and P19-061405 strains adapted to axenic culture conditions were significantly smaller than that of *E. histolytica* HM-1:IMSS. *E. histolytica* trophozoites obtained from intestinal or liver lesions are generally larger than those found in cultures (Martínez-Palomo, 1993), but the *E. nuttalli* trophozoites observed in liver in this study seemed to be as small as those in culture. It is unclear whether the size difference influences the overall characteristics of *E. nuttalli*, but this difference has also been found in a comparison of *E. histolytica* and *E. histolytica*-like Laredo (*E. moshkovskii*) trophozoites (Diamond, 1968; López-Revilla and Gómez-Domínguez, 1988).

The liver damage produced in hamsters inoculated with *E. nuttalli* trophozoites was similar to that described in the same species challenged with *E. histolytica* trophozoites (Tsutsumi *et al*. 1984). This suggests similar physiopathological mechanisms of tissue damage, including an important role of lysed inflammatory cells (Perez-Tamayo *et al*. 1991; Tsutsumi and Shibayama, 2006). Ultrastructural analysis of the amoeba-inflammatory cell interaction has shown that *E. nuttalli* carries a similar virulent capacity to destroy target cells, including lysis of inflammatory cells and endocytosis, despite the smaller size of trophozoites of the *E. nuttalli* NASA6 strain compared to the *E. histolytica* HM-1:IMSS strain (Tsutsumi and Martínez-Palomo, 1988).

If *E. nuttalli* is able to infect humans, it may be of concern as a zoonotic hazard. Therefore, a stool examination was performed on the 4 keepers taking care of the monkeys in the park. No parasites were detected from samples by direct microscopy or in culture in Tanabe-Chiba medium (data not shown). It remains possible that *E. nuttalli* is infective in humans, but *Entamoeba* with the zymodeme pattern of *E. nuttalli* has not been detected in more than 2500 separate isolations characterized to date (Sargeaunt, 1988). However, the results in this study show that the *E. nuttalli* NASA6 strain isolated from Japanese macaque is potentially virulent, and we suggest that *E. nuttalli* should be recognized as a common parasite in macaques and should be discriminated from *E. histolytica* and *E. dispar* in routine analysis.

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REFERENCES

Castellani, A. (1908). Note on a liver abscess of amoebic origin in a monkey. *Parasitology* **1**, 101–102.

Chu, J. H., Lin, Y. S. and Wu, H. Y. (2007). Evolution and dispersal of three closely related macaque species, *Macaca mulatta*, *M. cyclopis*, and *M. fuscata*, in the eastern Asia. *Molecular Phylogenetics and Evolution* **43**, 418–429.

Clark, C. G. (1995). Axenic cultivation of *Entamoeba dispar* Brumpt 1925, *Entamoeba insolita* Geiman and Wichterman 1937 and *Entamoeba ranarum* Grassi 1879. *Journal of Eukaryotic Microbiology* **42**, 590–593.

Diamond, L. S. (1968). Techniques of axenic cultivation of *Entamoeba histolytica* Schaudinn, 1903

https://doi.org/10.1017/S0031182009990576 Published online by Cambridge University Press

and *E. histolytica*-like amebae. *Journal of Parasitology* **54**, 1047–1056.

Diamond, L. S. and Clark, C. G. (1993). A redescription of *Entamoeba histolytica* Schaudinn, 1903 (Emended Walker, 1911) separating it from *Entamoeba dispar* Brumpt, 1925. *Journal of Eukaryotic Microbiology* **40**, 340–344.

Diamond, L. S., Harlow, D. R. and Cunnick, C. C. (1978). A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **72**, 431–432.

Espinosa-Cantellano, M., Gonzáles-Robles, A., Chávez, B., Castañón, G., Argüello, C., Lázaro-Haller, A. and Martínez-Palomo, A. (1998). *Entamoeba dispar*: ultrastructure, surface properties and cytopathic effect. *Journal of Eukaryotic Microbiology* **45**, 265–272.

Ghosh, S., Frisardi, M., Ramirez-Avila, L., Descoteaux, S., Sturm-Ramirez, K., Newton-Sanchez, O. A., Santos-Preciado, J. I., Ganguly, C., Lohia, A., Reed, S. and Samuelson, J. (2000). Molecular epidemiology of *Entamoeba* spp.: evidence of a bottleneck (Demographic sweep) and transcontinental spread of diploid parasites. *Journal of Clinical Microbiology* **38**, 3815–3821.

Hayasaka, K., Fujii, K. and Horai, S. (1996). Molecular phylogeny of macaques: implications of nucleotide sequences from an 896-base pair region of mitochondrial DNA. *Molecular Biology and Evolution* **13**, 1044–1053.

Hegner, R. and Schumaker, E. (1928). Some intestinal amoebae and flagellates from the chimpanzee, three-toed sloth, sheep and guinea-pig. *Journal of Parasitology* **15**, 31–37.

Kobayashi, S., Imai, E., Haghighi, A., Khalifa, S. A., Tachibana, H. and Takeuchi, T. (2005). Axenic cultivation of *Entamoeba dispar* in newly designed yeast extract-iron-gluconic acid-dihydroxyacetone-serum medium. *Journal of Parasitology* **91**, 1–4.

López-Revilla, R. and Gómez-Domínguez, R. (1988). Trophozoite and nuclear size, DNA base composition, and nucleotide sequence homology of several *Entamoeba* strains in axenic culture. *Parasitology Research* **74**, 424–430.

Martínez-Palomo, A. (1993). Parasitic amebas of the intestinal tract. In *Parasitic Protozoa*, 2nd Edn., (ed. Kreier, J. P. and Baker, J. R.), Vol. 3, pp. 65–141. Academic Press, San Diego, USA.

McLean, I. W. and Nakane, P. K. (1974). Periodate-lysine-paraformaldehyde fixative. A new fixation for immunoelectron microscopy. *Journal of Histochemistry and Cytochemistry* **22**, 1077–1083.

Muriuki, S. M., Murugu, R. K., Munene, E., Karere, G. M. and Chai, D. C. (1998). Some gastro-intestinal parasites of zoonotic (public health) importance commonly observed in old world non-human primates in Kenya. *Acta Tropica* **71**, 73–82.

Neal, R. A. (1966). Experimental studies on *Entamoeba* with reference to speciation. *Advances in Parasitology* **4**, 1–51.

Perez-Tamayo, R., Martinez, R. D., Montfort, I., Becker, I., Tello, E. and Perez-Montfort, R. (1991). Pathogenesis of acute experimental amebic liver abscess in hamsters. *Journal of Parasitology* **77**, 982–988.

Rivera, W. L. and Kanbara, H. (1999). Detection of *Entamoeba dispar* DNA in macaque feces by polymerase chain reaction. *Parasitology Research* **85**, 493–495.

Robinson, G. L. (1968). The laboratory diagnosis of human parasitic amoebae. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **62**, 285–294.

Sargeaunt, P. G. (1988). Zymodemes of *Entamoeba histolytica*. In *Amebiasis : Human Infection by* Entamoeba histolytica (ed. Ravdin, J. I.), pp. 370–387. John Wiley & Sons, New York, USA.

Smith, J. M. and Meerovitch, E. (1985). Primates as a source of *Entamoeba histolytica*, their zymodeme status and zoonotic potential. *Journal of Parasitology* **71**, 751–756.

Suzuki, J., Kobayashi, S., Murata, R., Yanagawa, Y. and Takeuchi, T. (2007). Profiles of a pathogenic *Entamoeba histolytica*-like variant with variations in the nucleotide sequence of the small subunit ribosomal RNA isolated from a primate (De Brazza's guenon). *Journal of Zoo and Wildlife Medicine* **38**, 471–474.

Tachibana, H., Cheng, X. J., Kobayashi, S., Fujita, Y. and Udono, T. (2000). *Entamoeba dispar*, but not *E. histolytica*, detected in a colony of chimpanzees in Japan. *Parasitology Research* **86**, 537–541.

Tachibana, H., Cheng, X. J., Kobayashi, S., Matsubayashi, N., Gotoh, S. and Matsubayashi, K. (2001). High prevalence of infection with *Entamoeba dispar*, but not *E. histolytica*, in captive macaques. *Parasitology Research* **87**, 14–17.

Tachibana, H., Yanagi, T., Pandey, K., Cheng, X. J., Kobayashi, S., Sherchand, J. B. and Kanbara, H. (2007). An *Entamoeba* sp. strain isolated from rhesus monkey is virulent but genetically different from *Entamoeba histolytica*. *Molecular and Biochemical Parasitology* **153**, 107–114.

Takano, J., Narita, T., Tachibana, H., Shimizu, T., Komatsubara, H., Terao, K. and Fujimoto, K. (2005). *Entamoeba histolytica* and *Entamoeba dispar* infections in cynomolgus monkeys imported into Japan for research. *Parasitology Research* **97**, 255–257.

Takano, J., Narita, T., Tachibana, H., Terao, K. and Fujimoto, K. (2007). Comparison of *Entamoeba histolytica* DNA isolated from a cynomolgus monkey with human isolates. *Parasitology Research* **101**, 539–546.

Takano, J., Tachibana, H., Kato, M., Narita, T., Yanagi, T., Yasutomi, Y. and Fujimoto, K. (2009). DNA characterization of simian *Entamoeba histolytica*-like strains to differentiate them from *Entamoeba histolytica*. *Parasitology Research* **105**, (in the Press). doi:10.1017/S00436-009-1480-3

Tsutsumi, V., Mena-Lopez, R., Anaya-Velazquez, F. and Martinez-Palomo, A. (1984). Cellular bases of experimental amebic liver abscess formation. *American Journal of Pathology* **117**, 81–91.

Tsutsumi, V. and Martínez-Palomo, A. (1988). Inflammatory reaction in experimental hepatic amebiasis. An ultrastructural study. *American Journal of Pathology* **130**, 112–119.

Tsutsumi, V. and Shibayama, M. (2006). Experimental amebiasis: a selected review of some in vivo models. *Archives of Medical Research* **37**, 210–220.

Verweij, J. J., Polderman, A. M. and Clark, C. G. (2001). Genetic variation among human isolates of uninucleated cyst-producing *Entamoeba* species. *Journal of Clinical Microbiology* **39**, 1644–1646.

Verweij, J. J., Vermeer, J., Brienen, E. A., Blotkamp, C., Laeijendecker, D., van Lieshout, L. and Polderman, A. M. (2003). *Entamoeba histolytica* infections in captive primates. *Parasitology Research* **90**, 100–103.

Wenyon, C. M. (1965). *Protozoology : A Manual for Medical Men, Veterinarians and Zoologists*. Hafner Publishing Company, New York, USA.

World Health Organization (1997). Amoebiasis. *Weekly Epidemiological Record* **72**, 97–99.

https://doi.org/10.1017/S0031182009990576 Published online by Cambridge University Press