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ESTABLISHMENT OF A CONTINUOUS CULTURE SYSTEM FOR *ENTAMOEBA MURIS* AND ANALYSIS OF THE SMALL SUBUNIT rRNA GENE

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Summary:

We established a culture system for *Entamoeba muris* (MG-EM-01 strain isolated from a Mongolian gerbil) using a modified Balamuth's egg yolk infusion medium supplemented with 4 % adult bovine serum and *Bacteroides fragilis* cocultured with *Escherichia coli*. Further, encystation was observed in the culture medium. The morphological characteristics of *E. muris* are similar to those of *Entamoeba coli* (*E. coli*); moreover, the malic isoenzyme electrophoretic band, which shows species-specific electrophoretic mobility, of *E. muris* had almost the same mobility as that observed with the malic isoenzyme electrophorectic band of *E. coli* (UZG-EC-01 strain isolated from a gorilla). We determined the small subunit rRNA (SSU-rRNA) gene sequence of the MG-EM-01 strain, and this sequence was observed to show 82.7 % homology with that of the UZG-EC-01 strain. Further, the resultant phylogenetic tree for molecular taxonomy based on the SSU-rRNA genes of the 21 strains of the intestinal parasitic amoeba species indicated that the MG-EM-01 strain was most closely related to *E. coli*.

KEY WORDS : *Entamoeba muris*, *Entamoeba coli*, dixenic culture, Balamuth's egg yolk infusion medium, SSU-rRNA gene, phylogenetic analysis.

Résumé : ÉTABLISSEMENT D'UN SYSTEME DE CULTURE SUCCESSIVE POUR *ENTAMOEBA MURIS* ET ANALYSE DU GÈNE CODANT POUR LA PETITE SOUS-UNITÉ DE L'ARNr

*Un système de culture d'*Entamoeba muris *(souche MG-EM-01, isolée da la gerbille de Mongolie) a été établi en utilisant un milieu d'infusion de vitellus de Balamuth modifié, supplémenté avec 4 % de sérum bovin adulte et de* Bacteroides fragilis *en coculture avec* Eschericia coli. *L'enkystement s'est également présenté dans le milieu de culture. Les aspects morphologiques d'*E. muris *sont semblables à ceux d'*Entamoeba coli *et la bande isoenzymatique malique présentant une mobilité électrophorétique spécifique à l'espèce avait à peu près la mobilité d'*Entamoeba coli *(souche UZG-EC-01, isolée d'un gorille). La séquence du gène codant pour la petite sous-unité de l'ARNr (SSU-rRNA) de la souche MG-EM-01 a été déterminée et l'homologie de la séquence était également identique à 82,7 % de celle de la souche UZG-EC-01. L'arbre phylogénétique qui en résulte pour la taxonomie moléculaire basée sur les gènes SSU-rRNA de 21 souches d'espèces de parasites intestinaux amibiens indiquait également que la souche MG-EM-01 était étroitement liée à* E. coli.

MOTS CLÉS : Entamoeba muris, Entamoeba coli, *culture dixenic, milieu d'infusion de vitellus de Balamuth, petite sous-unité de l'ARNr, analyse phylogénétique.*

Entamoeba muris is a highly contagious intestinal protozoan parasite of laboratory mice, rats and other rodents; this species is morphologically similar to *Entamoeba coli* (*E. coli*) (Neal, 1950) and primarily proliferates and encysts in the caecum of mice (Lin, 1971).

In vitro culture of *E. muris* has been attempted (Neal, 1950; Simitch & Petrovitch, 1951; Smith *et al.*, 1985); however, trials to achieve successive culture have not been successful.

In the present study, we established a system for the stable and successive culture of an *E. muris* strain. Further, we attempted phylogenetic analysis of this strain to help in investigating its molecular taxonomy.

MATERIALS AND METHODS

E. muris (strain MG-EM-01) isolated from a Mongolian gerbil spontaneously infected in our laboratory was used to establish the culture system. An *E. coli* strain (UZG-EC-01) isolated from a gorilla in a zoo in Tokyo, Japan, was used as a reference. *Escherichia coli* and *Bacteroides fragilis* strains were isolated from the stool of a primate [DeBrazza's guenon (*Cercopithecus neglectus*)] and fresh human stool samples from a patient with intestinal amoebic colitis, respectively. The *E. coli* strains were maintained in a chemically defined medium (R medium) (Robinson, 1968), and the *B. fragilis* strains were maintained on trypticase, yeast extract and iron (TYI) broth (Diamond *et al.*, 1978); these strains were used as supplements for the culture system of *E. muris*.

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The egg yolk infusion medium of Balamuth's medium (Balamuth, 1946) was replaced with infusion of a liver concentrate with 4 % preheat-treated adult bovine serum (56° C for three hours). At least one day prior to *E. muris* culture, 0.1 ml of *E. coli* maintained in the R suspension was added from the stock culture preserved at 4° C (used within one month); however, 0.2 ml of a 1- to 3-day culture of *B. fragilis* maintained in TYI broth was added to the abovementioned culture at the time of *E. muris* primary culture. The primary culture of *E. muris* was performed by inoculating cysts obtained from the stool sample of a Mongolian gerbil; this inoculation was performed after killing the concomitant enteric bacteria with 0.1 N HCl for 60 min at 35.5° C, and subsequent inoculation with a newly designed excystation medium (0.25 % trypsin, 0.24 % gall powder, and lipase (840 units/ml) from *Chromobacterium viscosum* (Sigma-Aldrich Corp., St. Louis, MO, USA) in Hanks' balanced salt solution) for 60 min at 35.5° C.

For 120 hours, the number of trophozoites in 5 µl of the abovementioned culture was counted microscopically at 24-hours intervals, as described previously (Kobayashi *et al.*, 2005). The resistance of the cysts to osmotic pressure was confirmed using 0.05 % sarcosyl (Eichinger, 1997) over a 24-hours period. The viability of the cysts was assessed by double-fluorescence staining using acridine orange and ethidium bromide (Parks *et al.*, 1979). The number of cysts in 5 µl of the homologous amoeba suspension in 0.05 % sarcosyl, which was adjusted to the same volume as that of the culture medium, was counted microscopically in a manner similar to that described previously (Kobayashi *et al.*, 2005).

In order to characterize the trophozoites, we performed isoenzyme analyses (zymodeme) (Sargeaunt, 1988) of *E. muris* and *E. coli.*

The primers for amplification of the SSU-rRNA gene sequences of the *E. muris* (MG-EM-01) and *E. coli* (UZG-EC-01) isolates were designed on the basis of the two SSU-rRNA sequences of *E. coli* [IH:96/135 (AF149914) and HU-1:CDC (AF149915)] acquired from GenBank. Table I lists the three primer sets designed, namely, Ecoli1F/Ecoli1R, Ecoli2F/Ecoli2R and Ecoli3F/Ecoli3R. The polymerase chain reaction (PCR) amplification and the sequencing analysis of the PCR products of the SSU-rRNA genes derived from MG-EM-01 and UZG-EC-01 were performed as described previously (Suzuki *et al.*, 2008).

Analysis and multiple alignments of the acquired sequences of the SSU-rRNA genes of *E. muris* (MG-EM-01) and *E. coli* (UZG-EC-01) were performed by following the Yebis system for DNA Alignment, which uses a tree-based round-robin iterative algorithm (Hirosawa *et al.*, 1995). The phylogenetic tree was constructed using PhyML software package version 2.4.5 (Guindon and Gascuel, 2003) using maximum likelihood (ML) analysis and a general time-reversible (GTR) model to calculate genetic distances. The reliability of the branches of the tree of the GTR model was tested with bootstrap values obtained from 1,000 replications. The ML tree data file from PhyML was read, and the tree was constructed using MEGA software (Tamura *et al.*, 2007).

RESULTS

We have been successfully culturing *E. muris* for greater than 16 months now. Fig. 1 shows the growth and encystation kinetics of *E. muris* maintained in modified Balamuth's medium. Encystation of *E. coli* isolates was not observed in this medium. The mean sizes of trophozoites (33.3 ± 8.9 × 21.6 ± 5.6 µm) and cysts (21.6 ± 3.0 × 20.8 ± 2.3 µm) of *E. muris* in the culture medium were almost the same as those of *E. coli* trophozoites (32.8 ± 12.6 × 28.4 ± 7.6 µm) and cysts (21.6 ± 6.1 × 19.8 ± 3.8 µm). However, similar to the result reported by Neal (1950), a greater number of trophozoites and cyst nuclei with extremely eccentric karyosomes and thin peripheral chromatin layers were observed in *E. muris* compared to *E. coli.* Additionally, a large chromatin mass in the polar position of the karyosome in the nucleus of the *E. muris* cyst was frequently observed in the cysts reproduced in the *in vitro* culture (Fig. 2).

Figure 3 shows a representation of the zymodeme analyses to estimate malic enzyme (ME) and hexokinase (HK) levels. The electrophoretic mobility of the single band of *E. muris* ME was almost the same as that observed with *E. coli* ME. However, the electrophoretic

Primer name	Primer sequence (5' to 3')	Nucleotide position*
Ecoli1F (forward)	GTT GAT CCT GCC AGT ATT ATA TG	7-28
Ecoli1R (reverse)	ATA CCA TGC TTC ATC ATT C	841-859
Ecoli2F (forward)	GTA ATT CCA GCT CCA ATA GTC	617-637
Ecoli2R (reverse)	AAG TTC AAG TCT CGT TCG TTA TCG GA	1467-1492
Ecoli3F (forward)	TGA CTC AAC ACG GGA AAA CTT	1339-1359
Ecoli3R (reverse)	ATC CTT CCG CAG GTT CAC CTA C	2083-2104

* Nucleotide position was based on sequence of *Entamoeba coli* UZG-EC-01 Srain (AB444953).

Table I. – Oligonucleotide primers used for PCR assays in present study.

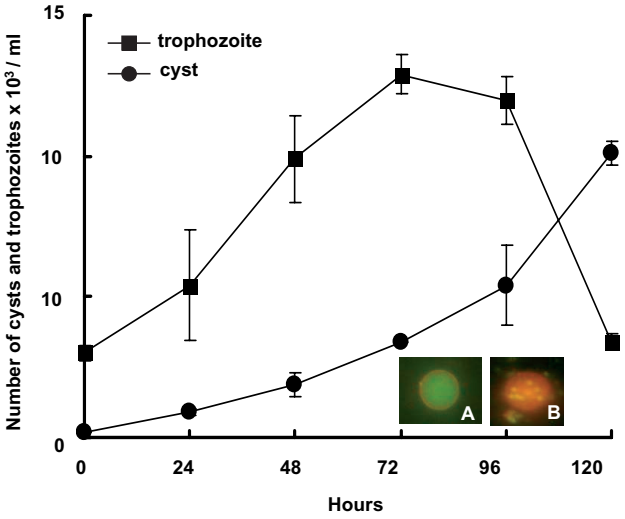

Fig. 1. – Growth kinetics of *Entamoeba muris* strain MG-EM-01 with reproduction of cysts in newly modified Balamuth's medium. The mean number of trophozoites and cysts in duplicate cultures are plotted. A and B, micrographs of cysts that were double-fluorochrome stained with acridine orange (AO) and ethidium bromide (EB). Live cysts (A) are stained with only AO (green colour); however, dead cysts (B) are stained with both AO and EB (red colour).

mobility of the double bands of *E. muris* HK showed a pattern that was different from that observed with *E. coli* HK, HM-1:IMSS clone 6 (ATCC 50527) (*E. histolytica*) HK, and AS 16 IR (*E. dispar*) HK (Kobayashi *et al.*, 2005) (Fig. 3). Under these electrophoretic conditions, distinguishable bands of phosphoglucomutase (PGM) and glucose phosphate isomerase (GPI) were observed neither in *E. muris* nor in *E. coli*.

On the basis of phylogenetic analysis, these four strains of *E. coli* and *E. muris* were included in the genus *Entamoeba* and the eight nuclei per cyst group were sister taxa in 100 % of the bootstrap resamplings (Fig. 4).

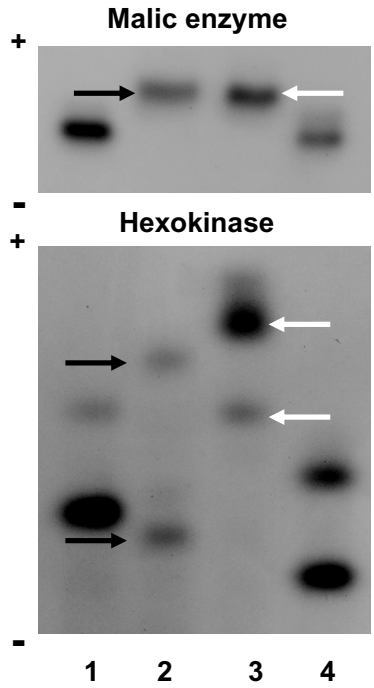

Fig. 3. – Isoenzyme pattern of malic enzyme and hexokinase of four strains of four different *Entamoeba* species. Lane 1, HM-1:IMSS clone 6 (*Entamoeba histolytica*); Lane 2, MG-EM-01 (*Entamoeba muris*); Lane 3, UZG-EC-01 (*Entamoeba coli*); Lane 4, AS 16 IR (*Entamoeba dispar*).

DISCUSSION

Escherichia coli, a facultative anaerobic bacterium, can produce anaerobic conditions that facilitate the culture of certain obligate anaerobic bacteria such as *B. fragilis*; this principle has been used in a coculture system with *Escherichia coli* in Robin-

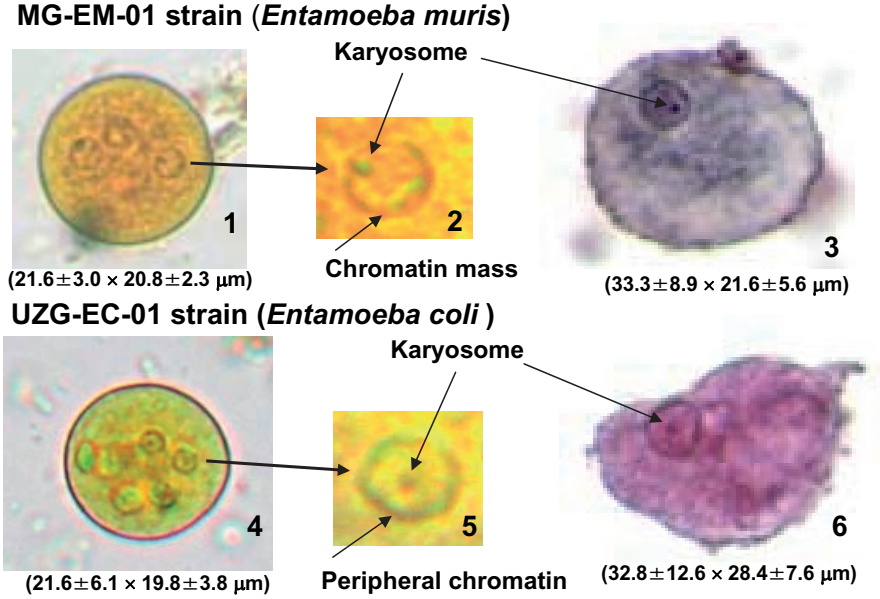

Fig. 2. – Micrographs of cysts and trophozoites of *Entamoeba muris* strain MG-EM-01 and *Entamoeba coli* strain UZG-EC-01. 1 and 4, cysts of MG-EM-01 (1) and UZG-EC-01 (4) strains stained with Lugol's iodine solution (Lugol). 2 and 5, nuclei of MG-EM-01 (2) and UZG-EC-01 (5) strains stained with Lugol. 3 and 6, trophozoites of MG-EM-01 (3) and UZG-EC-01 (6) strains stained with Kohn's chlorazol black E (Gleason *et al.*, 1965).

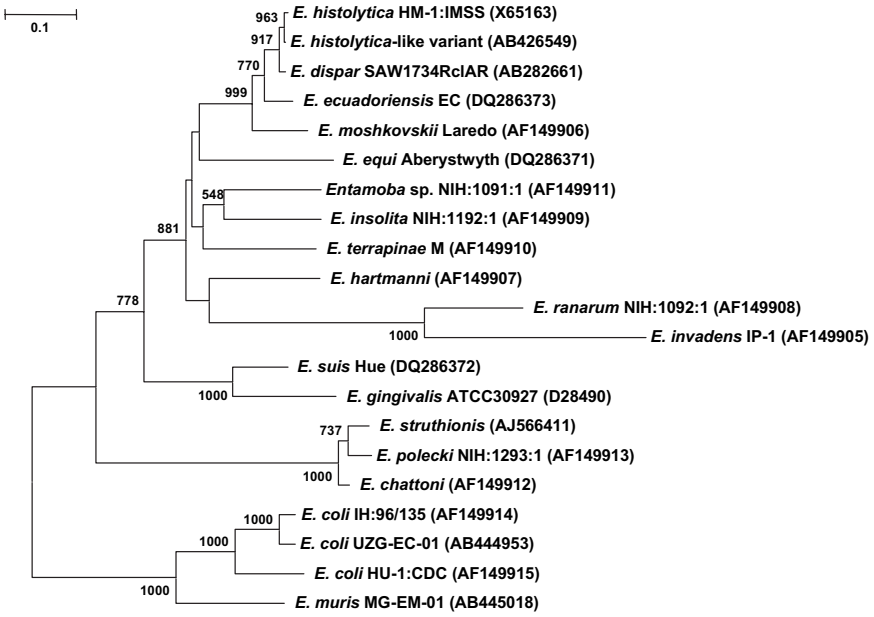

Fig. 4. – An unrooted phylogenetic reconstruction based on the SSU-rRNA gene sequences that explores the relationships among *Entamoeba* species is shown. A maximum likelihood (ML) tree derived using a general time reversible (GTR) model employing estimates of the proportion of invariable sites and the gamma distribution parameter of 0.423 and 0.169, respectively. Significant bootstrap support (> 500) from 1,000 replicates is indicated on the left of the supported node. The scale bar represents the evolutionary distance for the number of changes per site. The numbers within parentheses represent the corresponding GenBank accession numbers.

son's medium (Robinson, 1968). Some obligate anaerobic bacteria such as *Fusobacterium symbiosum* (*Clostridium symbiosum* ATCC 14940) (Diamond, 1983) can promote the growth of human parasitic *Entamoeba* isolates. Therefore, an isolate of *B. fragilis* that demonstrated a growth-promoting effect on the wild isolates of *E. histolytica* and *E. coli* in Balamuth's egg yolk infusion medium (data not shown) was used as a supplement for the culture of *E. muris;* thereafter, a successful culture system for *E. muris* was established for the first time. Phylogenetic analysis of the SSU-rRNA gene sequence of the *E. muris* (MG-EM-01) isolates, although derived from only one strain, suggested that *E. muris* is phylogenetically similar to *E.coli* that produced cysts with eight nuclei. The correlation between phylogenetic propinquity and the number of nuclei observed in the *Entamoeba* species possessing four nuclei per cyst has been previously reported (Silberman *et al.*, 1999; Clark *et al.*, 2006).
The established culture system continues to be dixenic. However, it enabled the analysis of the biological and molecular characteristics of an *E. muris* strain.

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