**Establishment of a Continuous Culture System for Entamoeba muris and Analysis of the Small Subunit rRNA Gene**

KOBAYASHI S.*, SUZUKI J.** & TAKEUCHI T.*

**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 electrophoretic band of E. coli. 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 parasite 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.

**Résumen :** Établissement d’un système 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 Escherichia 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*.
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

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

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

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

**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-
son’s medium (Robinson, 1968). Some obligate anaerobic bacteria such as *Fusobacterium symbiosis* (*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.

**ACKNOWLEDGEMENTS**

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