Genetic Variation among Human Isolates of Uninucleated Cyst-Producing Entamoeba Species

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Twelve human infections with Entamoeba spp. producing uninucleated cysts were studied. DNA was extracted from infected feces and used to amplify part of the ameba small-subunit rRNA gene. Sequence analysis identified four distinct types of Entamoeba, all of which are related to Entamoeba polecki and E. chattoni and two of which have not been reported previously. Whether these genetic types represent different species is unclear. We propose that the agent of all human infections with uninucleated cyst-producing Entamoeba species be reported as “E. polecki-like.”

Human cases of infection with the uninucleated cyst-producing Entamoeba species referred to as Entamoeba polecki are considered to be rare (2, 4), except in Papua New Guinea, where prevalence rates as high as 30% are reported (1, 5), and are often associated with contact with pigs. However, eight cases of human infection with a uninucleated cyst-producing Entamoeba species have been reported; these cases resulted from contact with monkeys, and the agent was identified as E. chattoni (6). The taxonomic status of these uninucleated Entamoeba species over the years has been confusing. They have been identified in various domestic and other animals and have species-specific diagnosis of E. histolytica or E. dispar. Furthermore, PCR–soluble hybridization enzyme-linked assay reactions for E. histolytica and E. dispar in these samples were negative. We classified such cysts as non-E. histolytica/non-E. dispar cysts, possibly E. polecki or E. chattoni. To confirm the morphological findings, we designed PCR primers based on the known small-subunit rRNA gene sequences for E. polecki and E. chattoni (GenBank accession no. AF149913 and AF149912) such that DNA should be amplified for E. polecki or E. chattoni specifically. The E. polecki-specific primer set consisted of forward primer Epo1 (5′-TCG ATA TTT ATA TTG ATT CAA ATG-3′) and reverse primer Epo2 (5′-CCT TTC TCC TTT TTT TAT ATT AG-3′), and the E. chattoni-specific primer set consisted of forward primer Echat1 (5′-AGG ATT GTT TGT ATA ACA AGT TC-3′) and reverse primer Echat2 (5′-TAA ATA ACC TTT CTC CTT TTA CT-3′).

Amplification reactions were performed in a volume of 40 μl containing PCR buffer (10 mM Tris-HCl, [pH 9.0], 1.5 mM MgCl2, 50 mM KCl, 0.1% Triton X-100, and 0.01% [wt/vol] gelatin; HT Biotechnology, Cambridge, United Kingdom), each deoxynucleoside triphosphate at 200 μM, 25 pmol of each specific primer, 1 U of Taq polymerase (SuperTaq HC; HT Biotechnology), and 2 μl of the DNA sample. Amplification consisted of 5 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C; and finally 2 min at 72°C. Only 1 sample was positive with the E. polecki primers, and 2 samples were positive with the E. chattoni primers; the other 12 samples remained negative.

To prove that Entamoeba species were indeed present in the negative samples, we designed general Entamoeba primers based on the small-subunit rRNA gene sequences for E. polecki, E. chattoni, E. dispar, E. histolytica, E. hartmanni, and E. coli (GenBank accession no: AF149913, AF149912, Z49256, X64412, AF49906, and AF149915, respectively). Forward prim-
er Entam1 (5'-GGT GAT CCT GCC AGT ATT ATA TG-3') and reverse primer Entam2 (5'-CAC TAT TGG AGC TGG AAT TAC-3') were chosen from conserved regions so that DNA of all Entamoeba species would be amplified. Amplification was performed under the conditions described above. In all 15 samples with uninucleated cysts, the expected amplicon of approximately 550 bp was produced. For further analysis, sequencing of the products was performed using the BigDye
A large genetic distance exists between the uni-, tetra-, and octanucleated cyst-forming *Entamoeba* species, as described by Silberman et al. (7). As shown in the phylogenetic tree, all 12 of our sequences cluster with the *E. polecki* and *E. chattoni* reference sequences and are widely separated from *E. coli* on one hand and from *E. histolytica*, *E. dispar*, and *E. hartmanni* on the other. Interestingly, within the uninucleated sequence group, four variants are clearly distinguishable. This is already evident in the alignment and is supported by the phylogenetic tree. The sequence from the sample that produced an amplicon with the *E. polecki*-specific primers was almost identical to the corresponding region of the GenBank sequence for *E. polecki*. The two samples that produced amplicons with the *E. chattoni* primers were almost identical to the corresponding region of the *E. chattoni* GenBank sequence. It is likely that the other 12 samples were initially negative for the *E. chattoni*- and *E. polecki*-specific reactions due to sequence divergence in one or more of the primer-binding sites.

We have shown that there are (at least) four genetic types of uninucleated cyst-producing *Entamoeba* species that infect humans. Unfortunately, any mixed infections of uninucleated *Entamoeba* species with *E. histolytica* or *E. dispar* would have been missed in this study because only *E. histolytica*/*E. dispar*-negative samples were used. Therefore, the prevalence of the infection cannot be accurately calculated.

At present we do not know whether the *E. chattoni*-like infections originated from contact with monkeys or whether the *E. polecki*-like infections came from pigs. What is clear, however, is that humans can undoubtedly be infected with uninucleated *Entamoeba* species and that more genetic variability exists within this group than previously has been recognized in human infections. Whether the two new uninucleate sequence types correspond to previously described species in other animals remains unknown, as material for comparison has not been available. As there is no consensus on the use of ribosomal sequences to define new species of protozoa, until the species involved can be identified or named, we suggest that the agent of all uninucleated *Entamoeba* infections in humans be reported as "*E. polecki*-like."

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