High Seroprevalence of Encephalitozoon Species in Immunocompetent Subjects

Tom van Gool, J. C. M. Vetter, B. Weimayr,* A. Van Dam, F. Derouin, and J. Dankert

Encephalitozoon species are important pathogens in human immunodeficiency virus–infected patients. However, in immunocompetent persons, little is known about Encephalitozoon infections, mainly because of the lack of reliable diagnostic tests. To improve diagnosis, three serologic techniques that use Encephalitozoon intestinalis as antigen were developed: an ELISA, an immunofluorescence technique (IFAT), and a counterimmunoelectrophoresis (CIE) method. The serologic response against E. intestinalis was studied in sera from 300 Dutch blood donors and 276 pregnant French women. For confirmation of specificity, sera from 150 subjects with various infectious and noninfectious diseases were examined. ELISA, IFAT, and CIE were specific for microsporidia infections, and IFAT and CIE were specific for Encephalitozoon infections. High antibody titers against Encephalitozoon organisms were found in 24 (8%) of 300 Dutch blood donors and in 13 (5%) of 276 pregnant French women. The high seroprevalence against Encephalitozoon species in Dutch blood donors and French women suggests that Encephalitozoon infection is common in immunocompetent subjects.

Microsporidia of the genus Encephalitozoon have recently gained wide interest as important opportunistic parasites among immunocompromised patients [1–3]. In persons infected with the human immunodeficiency virus (HIV), microsporidial disease most frequently is due to Encephalitozoon intestinalis (formerly Septata intestinalis) and Encephalitozoon hellem [1, 3, 4]. Both species may disseminate to various organs, causing hepatitis, nephritis, sinusitis, keratoconjunctivitis, and bronchopneumonia [1, 3]. A third Encephalitozoon species, E. cuniculi, is mainly an animal pathogen and is only sporadically observed in humans [5].

Among immunocompetent, HIV-negative subjects, only 2 cases of Encephalitozoon infection, both children with neurologic disorders, have been described [6, 7]. Although Encephalitozoon spores were found in their cerebral spinal fluid and urine specimens, spore shedding occurred intermittently and only for a short period. Therefore, serologic methods could be of major importance for diagnosis of Encephalitozoon infections in immunocompetent subjects.

We recently isolated and cultured in vitro E. intestinalis from HIV-infected subjects [8]. This enabled us to use E. intestinalis as antigen and to initiate three new serologic techniques for the study of Encephalitozoon infections in humans.

**Subjects and Methods**

**Sera sources.** The serologic response to E. intestinalis was studied in 300 healthy Dutch blood donors (179 male, 121 female) and 276 healthy French pregnant women using consecutively collected sera. Sera from 150 patients with various parasitologic, viral, bacterial, or fungal infections were studied as controls. There were 10 subjects each who had malaria, leishmaniasis, toxoplasmosis, amebiasis, infectious mononucleosis, hepatitis A, hepatitis B, syphilis, borreliosis, Mycoplasma infection, and aspergillosis; 15 had cytomegalovirus infection. In addition, sera from 15 patients with rheumatoid factor and from 10 patients with antinuclear antibodies were examined.
Antigen preparation for ELISA and counter immunoelectrophoresis (CIE). E. intestinalis AMC1 was cultured from a stool specimen of an AIDS patient [8]. The spores were stored at 4°C, pooled, and washed three times with 140 mM PBS (pH 7.2; 2600 g, 4°C, 20 min). In total, 2 × 10⁶/mL spores were resuspended in 10 mL of PBS/distilled water (1:3) and sonicated in a cell disrupter (B15; Branson Ultrasonics, Danbury, CT) on ice at 90 W at 50% duty cycle for 40 min. After centrifugation (2600 g, 4°C, 20 min), the sonicate supernatant was collected and freeze-dried overnight. The antigen was resuspended in 1.5 mL of PBS. The protein content (according to the technique of Lowry) was 2.5–4.0 mg/mL. Antigen preparations of human microsporidian isolates E. hellem and Vittataforma cornea (formerly Nosema corneum), gifts of E. S. Didier (Tulane University, New Orleans), were prepared similarly.

ELISA. We diluted 10 μL of antigen in 50 mM sodium carbonate buffer (pH 9.6); 2.5–4.0 ng of protein/well was coated overnight at 4°C onto wells of thin-wall polycarbonate plates (Thermowell 192; Corning Costar, Cambridge, MA). Plates were then postcoated with 1 μL of 1% dried chicken egg white (Sigma, St. Louis) in 100 mM PBS containing 0.05% Tween 20 (PBS/CEW/T) at 37°C for 30 min. After three washes with PBS/T, 10 μL of 2-fold dilutions (1:50 until 1:6400) of serum samples diluted with PBS/CEW/T was added to each well. Each sample was tested in duplicate. As a control for aspecific binding, conjugate wells were filled with 10 μL of PBS/CEW/T. After incubation for 1 h at 37°C and five washes with PBS/T, 10 μL of goat anti-human IgG (Fc) antiserum conjugated with horseradish peroxidase (Nordic, Tilburg, Netherlands) diluted 1/1000 in PBS/CEW/T was incubated with each well for 1 h at 37°C. Plates were washed five times with PBS/T and incubated at room temperature for 30 min with 10 μL of 1 mg/mL 5-amino-2-salicylic acid (Merck-Schuchardt, Darmstadt, Germany) in 10 mM phosphate buffer (pH 5.95) containing 0.15 mM EDTA. Thirty minutes after the addition of 0.03% H₂O₂, the red-brown reaction product was scored visually. The titer of a serum sample was the reciprocal of the highest dilution showing a darker color than the control wells. For ELISAs with E. hellem and V. cornea antigens, the appropriate antigen preparation of both species was coated on Thermowell plates.

CIE. CIE gels were prepared by dissolving 0.2 g of purified agar (L28; Oxoid, Basingstoke, UK) under heat in 25 mL of Veronal buffer (0.01 M diethylbarbituric acid, 0.05 M diethylbarbituric acid sodium salt, pH 8.7). The agar was poured over 0.2 mm Gelbond film (110 × 125; Bio-Rad, Richmond, CA) and left for 60 min to solidify. Sample wells (4-mm diameter, 9 mm apart) were punched in two opposing rows. In the anode wells, 16 μL of undiluted serum was pipetted, and after a 10-min prerun, 16 μL of the antigen preparation (0.5 mg/mL) was applied to the cathode well. Immunoelectrophoresis was done at 70 V and 20 mA in Veronal buffer for 80 min. After overnight precipitation at 4°C, the gels were washed in 0.9% NaCl, dried, stained with Coomassie blue, destained, air-dried, and examined for precipitation lines. CIE for E. hellem and V. cornea was done with appropriate antigen preparations.

Immunofluorescence technique (IFAT). RK 13 cells infected with E. intestinalis AMC1 growing in flat-bottomed flasks were harvested and forced through a needle for separation (21 G; 20.8 × 50 mm). We applied 10 μL of the cell suspension to each well of a 12-well glass slide. After incubation at 37°C overnight in a humid atmosphere with 5% CO₂, the wells were washed in PBS (37°C), air-dried, fixed in cold acetone (−20°C) for 20 min, and stored at 4°C until use. Glass slides were washed with distilled water for 5 min. Each serum sample was diluted 2-fold in PBS (1:20–1:320). For each slide, 10 μL of diluted serum (10 μL of PBS for the conjugate control) was incubated for 30 min at 37°C in a humid atmosphere and then washed twice with PBS, rinsed with distilled water, and air-dried. Anti-human IgG (Fc and Fab) fluorescein isothiocyanate−labeled conjugate (10 μL; well, 1:60 diluted; Nordic) was incubated with the slides at 37°C for 30 min. Slides were washed twice with PBS and once with distilled water, air-dried, and covered with mounting medium (H-1000 Vectorshield; Vector Laboratories, Burlingame, CA) and a coverslip.

Slides were examined at ×630 magnification under a Leitz fluorescence microscope. For IFAT with E. hellem and V. cornea, cells infected by either of these Microsporidia species and coated onto glass slides were used as antigen. Control glass slides were coated with uninfected cells.

Adsorption experiments. Serum adsorption experiments were done with antigen preparations of E. intestinalis, Toxoplasma gondii, Entamoeba histolytica, and Escherichia coli. Antigen preparations were prepared by sonication and had a protein content of 2.5–3.0 mg/mL. For adsorption, 100 μg of the antigen preparation was added to 5 μL of prediluted serum samples (1:5 in PBS), which subsequently were shaken at 4°C for 18 h and centrifuged (12,000 g, 5 min). Measurements were made using the supernatants.

Statistical analysis. For data analysis, Spearman’s correlation coefficient was calculated for the ELISA and IFAT in Dutch blood donors. If the IFAT titer was <1:20, a titer of 19 was used for analysis. Similarly, if an ELISA titer was <50, the titer was set at 49. An unpaired t test was used to calculate differences in age between the infected and noninfected Dutch blood donors. The χ² test was used for comparison of proportions.

Results

Criteria for seropositivity. Sera from 300 blood donors were first examined by ELISA using E. intestinalis antigen. Results by ELISA titers varied from <50 (no reaction) to >1600. High ELISA titers (≥800) were observed in 28 sera. To assess the relation between the ELISA results and the other techniques and to define a criterion for seropositivity, 113 of the 300 sera were subjected to IFAT and CIE. The 113 sera included 18 samples with ELISA titers of <50, 50, 100, 200, 400, or 800 (n = 108) and all 5 sera with ELISA results ≥1600.

In IFAT, immunofluorescence was directed against the filament and the anchoring disk (IFAT⁵) (figure 1). Immunofluorescence of the spore wall was not observed. We found a high correlation between the ELISA IgG titers and IFAT⁵ titers (r = .92). A positive CIE was observed in 21 of 23 sera with high ELISA titers (≥800) and in 28 of 36 sera with high IFAT⁵ titers (≥80), suggesting a good correlation between CIE with ELISA and IFAT⁵.

A serum specimen was regarded as Encephalitozoon-sero-positive if the ELISA titer was ≥800, the IFAT⁵ titer was ≥80, and there was a precipitation line in CIE.
**Specificity of tests.** In the ELISA and IFAT, no reaction was observed with the conjugate control. In sera with high ELISA titers, adsorption with *E. intestinalis* antigen abolished reactivity in ELISA and in the IFAT method. Adsorption with antigen of *T. gondii*, *E. histolytica*, or *E. coli* did not alter the original ELISA or IFAT findings. In CIE, the sera showed no precipitation lines when *T. gondii* or *E. histolytica* antigen was used.

Sera with high (≥800) antibody titers against *E. intestinalis* also reacted in the ELISA when *E. hellem* or *V. corneae* antigen was used. In the IFAT and CIE methods, cross-reactivity was observed with *E. hellem* antigen, but not with *V. corneae* antigen, indicating specificity at the genus level by IFAT<sup>f</sup> and CIE.

Twelve of 150 sera from patients with various infectious and noninfectious diseases had ELISA titers ≥800; of these, 6 had IFAT<sup>f</sup> titers ≥80. These 6 sera (4%) were from patients with leishmaniasis (1 patient), infectious mononucleosis (1), hepatitis A (1), positive rheumatoid factor (1), and aspergillosis (2). Adsorption of these sera with *E. intestinalis* antigen abolished the ELISA and IFAT<sup>f</sup> titers.

**Seroprevalence of *Encephalitozoon* infection in Dutch blood donors and pregnant French women.** Among 300 blood donors, 28 had high ELISA titers. Of these, 24 (8%) fulfilled the criterion for *E. intestinalis* seropositivity (table 1). The other 4 sera with a raised ELISA titer (800) had a low IFAT titer (40), and 3 were negative by CIE. The seroprevalence among male and female blood donors was 15 (8.4%) of 179 and 9 (7.4%) of 121, respectively. Mean age of seropositive male and female blood donors did not differ (*t* test, *P* ≥ .06).

Thirteen sera (5%) from 276 pregnant French women met the criterion for seropositivity (table 1). Twelve other sera had raised ELISA titers (800). All of these were negative by CIE, and 10 had low IFAT<sup>f</sup> titers (<80).

All *E. intestinalis*-seropositive serum specimens from Dutch blood donors and pregnant French women were adsorbed with *E. intestinalis* antigen and always yielded low ELISA (<50) and IFAT<sup>f</sup> (<20) titers. The seroprevalence in the Dutch and French study groups was not different ($\chi^2$ test, *P* = .11).

Age-specific prevalences among the combined study populations increased significantly from 0 among persons 10–19 years old to 9.6% in those 40–49 years old (test for trend, *P* = .02).

**Discussion**

Using stringent criteria for seropositivity, we observed a high seroprevalence of antibodies against *Encephalitozoon* organisms among Dutch blood donors (8%) and pregnant French women (5%).

In earlier serologic studies in which *E. cuniculi* antigen was used, anti-*Encephalitozoon* antibodies were observed among persons who had visited tropical countries but seldom among healthy blood donors [9, 10]. In the previous ELISA and IFAT studies, antibodies reacting with the spore wall were measured, but not those reacting with the filament [9–12]. Because antibodies directed against the spore wall of *E. cuniculi* show cross-reactivity with spore-wall antigens of other microsporidia...
Controversy exists about the specificity of the tests used in the earlier studies [14]. In the present study, most sera with high ELISA titers in the IFAT technique showed a strong immunofluorescence against the filament and its anchoring disk (IFAT5). In animals with Encephalitozoon infection, the filament also is a major target for specific antibody response [15]. The good correlation between the IFAT5 and CIE suggests that, most likely, antigens on the filament were involved in the precipitation reaction. Immunofluorescence of the spore wall of E. intestinalis was not observed in Dutch blood donors but occurred in sera from 5 French women (data not shown). When tested in the IFAT with V. corneae as antigen, cross-reactivity was observed in sera from 3, confirming earlier studies that antibodies against spore-wall antigens can cross-react with different microsporidia genera [13].

Because the phylum Microspora contains over 80 genera, of which 4 are known to infect humans [14], it is important to ascertain whether the antibody response observed in our tests was elicited by E. intestinalis, the parasite under study. Therefore, we applied antigens from a closely related species (E. hellem) and a taxonomically more distinct species (V. corneae), both isolated from humans. When E. hellem was used as antigen, cross-reactivity was observed by ELISA, IFAT, and CIE. However, no cross-reactivity with V. corneae antigen was noted with IFAT and CIE, indicating that these techniques are specific at the genus level. Enterocytozoon bieneusi is frequently found in HIV-positive persons; however, the prevalence of E. bieneusi infection among immunocompetent persons and the production of specific antibodies in the course of such infection is unknown. With the observed genus specificity of our IFAT6 and CIE techniques, it is unlikely that antibodies against E. bieneusi, if present, interfered with the results of our study.

A positive ELISA, IFAT, and CIE result strongly suggests that the antibodies measured in the Dutch and French study groups are specific for Encephalitozoon organisms. Adsorption tests and the results from studies using sera from 150 patients with various infectious and noninfectious diseases were in agreement with this finding. The seroprevalence of 4% among these patients is in accordance with the seroprevalence observed among healthy Dutch blood donors.

With our techniques, antibodies against E. intestinalis and E. hellem could not be differentiated. For the moment, this seems of less importance because infections due to both species show considerable overlap in clinical symptomatology and can be treated effectively with albendazole [1, 3].

Our study does not provide information as to whether sera with low ELISA and IFAT titers also reflect Encephalitozoon infection, since precise cutoff levels for ELISA and IFAT could not be established. Follow-up studies among immunocompetent subjects with parasitologically proven Encephalitozoon infection are required for insight into the natural course of the IgG response and involvement of other isotypes in recent and past infections.

Only limited conclusions regarding the epidemiology of Encephalitozoon infection can be drawn from our data. The risk of infection seemed similar for male and female Dutch blood donors and pregnant French women. Peak prevalences were in the fourth and fifth decade of life among Dutch blood donors, suggesting the infection is infrequent in childhood. However, our study had limited power to detect small differences. Further cross-sectional and longitudinal studies are needed to elucidate the age pattern of infection and to identify risk groups.

Whether infection with Encephalitozoon in immunocompetent subjects is associated with symptomatic disease needs to be defined. Observations in humans and animals suggest that Encephalitozoon infection is associated with neurologic and renal disorders [6, 7, 14]. In addition, Encephalitozoon parasites are well known for their ability to cross the placenta, resulting in severe congenital infection in animals [14]. The new serologic methods described are important tools to study the epidemiology and pathogenicity of Encephalitozoon infections in immunocompetent and immunocompromised humans.

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References


