Comparison of the Concentration of Encephalitozoon cuniculi Genotypes I and III in Inflammatory Foci Under Experimental Conditions

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Background: Microsporidia of the genus Encephalitozoon are usually associated with severe infections in immunodeficient hosts while, in immunocompetent ones, microsporidiosis produces minimal clinically apparent disease. Despite their microscopic size, microsporidia are capable of causing systemic infection within a few days. However, the mechanisms by which microsporidia reach target tissues during acute infection remain unclear. Out of four genotypes of Encephalitozoon cuniculi, only three are available for experimental studies, with E. cuniculi genotype II being the best characterized.

Methods: In the present study, we tested the association between inflammation induction in immunocompetent and immunodeficient mice and the presence of spores of E. cuniculi genotypes I and III in selected organs using molecular methods and compared the results with previously published data on E. cuniculi genotype II.

Results: We reported the positive connection between inflammation induction and the significant increase of E. cuniculi genotypes I and III occurrence in inflammatory foci in both immunocompetent BALB/c and immunodeficient severe combined immunodeficient (SCID) mice in the acute phase of infection. The induction of inflammation resulted in increased concentration of E. cuniculi of both genotypes in the site of inflammation, as previously reported for E. cuniculi genotype II. Moreover, our study extended the spectrum of differences among E. cuniculi genotypes by the variations in dispersal rate within host bodies after experimentally induced inflammation.

Conclusion: The results imply possible involvement of immune cells serving as vehicles transporting E. cuniculi towards inflammation foci. The elucidation of possible connection with pro-inflammatory immune responses represents an important challenge with implications for human health and the development of therapeutic strategies.

Keywords: Encephalitozoon cuniculi genotype I, Encephalitozoon cuniculi genotype III, inflammation, targeted migration
been detected in cats, dogs, marmots, and in immunosuppressed humans,\textsuperscript{3,41,42} causing disseminated encephalitozoonosis in the latter species. However, contrary to the first three genotypes, genotype IV is not available for in vitro culture and thus for laboratory experiments.

\textit{Encephalitozoon} species infect several cell types in mammalian hosts, including epithelial and endothelial cells, fibroblasts, macrophages, and astrocytes\textsuperscript{43} mainly in the gastrointestinal tract, kidney, brain, eyes, sinuses, and muscles.\textsuperscript{35,36,44–46} Recently, a possible involvement of immune cells connected with pro-inflammatory immune responses serving as vehicles transporting \textit{E. cuniculi} genotype II purposefully across the whole host body towards inflammation was suggested, based on the occurrence of microsporidia in inflamed tissues,\textsuperscript{47} and experimentally verified.\textsuperscript{48}

The present study was designed to compare the progress of concentration of \textit{Encephalitozoon cuniculi} genotypes I and III in sites of inflammation with previously published data on \textit{E. cuniculi} genotype II\textsuperscript{48} and to answer the question of whether individual genotypes of \textit{E. cuniculi} differ in dispersal within the host body following experimentally induced inflammation.

\textbf{Materials and Methods}

\textbf{Ethics Statement}

All experimental procedures complied with the law of the Czech Republic (Act No 246/1992 Coll., on the protection of animals against cruelty). The study design was approved by ethical committees at the Biology Centre of CAS, the State Veterinary Administration, and the Central Commission for Animal Welfare under protocol no. 35/2020.

\textbf{Mice}

Eight-week-old Severe Combined ImmunoDeficient (SCID) and immunocompetent (BALB/c) mice originally obtained from Charles River (Sulzfeld, Germany) were bred in plastic cages with sterilized wood-chip bedding situated in flexible film isolators (BEM Znojmo, Czech Republic) with HEPA filters. Mice were supplied with a sterilized diet (TOP-VELAZ Praha, Czech Republic) and sterilized water ad libitum.

\textbf{Parasites}

The spores of \textit{E. cuniculi} genotype I (lagomorph subtype, ATCC\textsuperscript{®}-50503TM) originally isolated from rabbit, OH, USA in 1978 (ATCC\textsuperscript{®} Product Sheet), and spores of \textit{E. cuniculi} genotype III originally isolated from pet rodents, Steppe lemmings (\textit{Lagurus lagurus}), CZE in 2014\textsuperscript{32} were grown in vitro in Green monkey kidney cells (VERO, line E6) maintained in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 2.5\% heat inactivated fetal bovine serum. Spores were harvested and purified from cells by centrifugation over 50\% Percoll (Sigma-Aldrich) at 1100\texttimes g for 30 min and washed three times in deionized water before storing in deionized water at 4\degree C supplemented with antibiotics (Sigma-Aldrich, 100 U/mL penicillin, 100 \mu g/mL streptomycin, and 2.5 \mu g/mL amphotericin B). The spores were washed with deionized water before use.

\textbf{Experimental Protocol}

Fifteen SCID and 27 BALB/c mice were per orally (P.O.) infected with the dose of \(10^7\) either \textit{E. cuniculi} genotype I or III spores in 0.2 mL of deionized water using intragastric gavage. The inflammation in experimental SCID and BALB/c was induced by the usage of 50 \mu L of Freund’s Incomplete Adjuvant (Sigma-Aldrich) inoculated in the acute phase of infection\textsuperscript{49} to the hind limb muscle. Mice of both strains injected intramuscularly with 50 \mu L of sterile phosphate buffered solution (PBS) at the same intervals were used as negative controls. The animal’s health, mortality, and morbidity were recorded at 12-hour intervals.

Three mice from each group were euthanized by cervical dislocation every seventh day post-infection (DPI), and sterile samples for molecular detection and histology were prepared from limb muscles and liver by the usage of a different pair of sterile dissection tools.
DNA Isolation
Tissue samples were homogenized by bead disruption using a FastPrep®–24 Instrument (MP Biomedicals, CA) and 0.5 mm glass beads (Biospec Products, Inc., Bartlesville, OK) at a speed of 5.5 m/s for 1 min. Total DNA was extracted using a commercially available column based isolation kit, DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany), according to the manufacturer’s instructions. Extracted DNA was stored at −20°C.

PCR Amplification
Nested PCR protocols were used for amplification of a partial sequence of 16S rRNA, using microsporidia-specific primers. DNA obtained from spores of *E. cuniculi* genotype II and ultrapure water was used as a positive and negative control, respectively. PCR products were evaluated by gel electrophoresis.

qRT-PCR
DNA samples from limb muscles and liver were processed using a quantitative RT-PCR protocol amplifying a 268-bp region of the 16S rRNA gene of *E. cuniculi* and a 137-bp region of murine β-actin as a housekeeping gene. Each run included negative controls consisting of unspiked specimens and diluent blanks. The total amount of spores in 1 g of individual tissue samples was calculated based on the number of β-actin copies in the tissue sample based on a method routinely established in the laboratory.

Statistical Analysis
Differences in microsporidia presence in inflammatory foci compared to the non-induced site were analyzed by Student’s *t*-tests and the conformity of the variances of the tested groups was verified by an F-test at a confidence level of α≤0.05. All computations were made using Statistica 6.0 software (StatSoft CR, Praha, Czech Republic).

Results
The application of Freund’s Incomplete Adjuvant elicited a local inflammatory immune response in all animals characterized by the formation of a purulent inflammatory focus in the hind limb muscle. On the other hand, no macroscopic changes were observed in the PBS-inoculated mice.

Induction of Inflammation in SCID Mice
In immunodeficient SCID mice, *E. cuniculi* genotypes I and III were detected in all screened tissue already prior to induction of inflammation (Figure 1A and B) with a higher spore burden in *E. cuniculi* genotype III infected mice, reaching up to 5.2×10² spores per gram of tissue.

In the control group of immunodeficient SCID mice infected with *E. cuniculi* genotype I (Figure 1A) and with no inflammation induction (injected with PBS only), no effect was observed and the number of spores increased continuously till the end of experiment 35 days post-infection (DPI), reaching up to 1.7×10⁴ spores per gram of tissue (Figure 1A).

After induction of inflammation of 7 DPI in the experimental group, in the non-induced leg, the spore burden was similar to the control group; however, a significant (*p*<0.05) increase in the number of spores in the induced right hind leg was observed, starting at 14 DPI and reaching up to 2.4×10⁵ spores per gram of tissue at 35 DPI, compared to the left hind leg, containing maximally 3.1×10⁴ spores per gram of tissue at 35 DPI. In addition, there was a significant difference (*p*<0.05) in spore burden in induced animals compared to the control mice, and the infection trend was more intense in induced animals (Figure 1A).

A similar situation was observed in SCID mice infected with *E. cuniculi* genotype III. There was no difference within control groups (Figure 1B), except that the spore burden in individual tissues was higher compared to *E. cuniculi* genotype I, reaching up to 4.7×10⁶ spores per gram of tissue at 35 DPI.

In the experimental group infected with *E. cuniculi* genotype III with induced inflammation, there was a one order shift toward the induced site starting at 14 DPI reaching 2.7×10², 2.1×10⁴, 2.1×10⁶, and 1.0×10⁷ spores per gram of tissue, compared to 1.5×10¹, 2.2×10³, 1.5×10⁵, and 1.3×10⁶ spores per gram of tissue in the control group (Figure 1B).
Surprisingly, a significant decrease in spore burden was observed in the non-induced site at 14 DPI (e.g., $1.5 \times 10^6$ spores per gram of tissue). Moreover, there was a significant difference ($p<0.05$) in spore burden in induced animals compared to the control mice starting from 21 DPI (Figure 1B). The infection trend was significantly more intense and reached a higher spore burden in the induced site compared to SCID mice infected with *E. cuniculi* genotype I (Figure 1A).

### Induction of Inflammation in BALB/c Mice

Also in immunocompetent BALB/c mice were *E. cuniculi* genotypes I and III, detected prior to induction of inflammation in all screened tissue samples at least in one animal per group (Figure 2A and B).

In the control group of immunocompetent BALB/c mice infected with *E. cuniculi* genotypes I and III, no differences in microsporidia distribution were observed and the number of spores was comparable in both legs, increased at the beginning of the infection, reaching up to $2.3 \times 10^2$ and $9.9 \times 10^1$ spores per 1 g of tissue, respectively, and followed by

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**Figure 1** Maximum value of *E. cuniculi* spores per 1 gram of tissue (number in white fields), frequency and spore burden of *E. cuniculi* spores per gram of tissue (colored squares; each square represents one mouse), and infection trend illustration (red fields) in immunodeficient SCID mice infected either with *E. cuniculi* genotype I (A) or *E. cuniculi* genotype III (B) and induced in the acute phase of infection. *Peroral infection 10^7* spores of *E. cuniculi* in 200 µL dH2O and intramuscular injection of PBS. *Peroral infection 10^7* spores of *E. cuniculi* in 200 µL dH2O and intramuscular injection of Freund's Incomplete Adjuvant; grey column, intramuscular injection of all animals in the group into the right thigh muscle; *significant difference between right and left leg; *significant difference between control and experimental group; colored squares, positive capture of microsporidia in the sample according to the quantity scale.
continuous disappearance and clearance of microsporidia in latter weeks of infection from muscles, namely 42 DPI (E. cuniculi genotype I) and 49 DPI (E. cuniculi genotype III) (Figure 2A and B).

The induction of inflammation in immunocompetent BALB/c mice infected with E. cuniculi genotype I during the acute phase of infection (7 DPI) resulted in the disappearance of microsporidia from the non-induced left hind leg and an increase of their concentration toward the induced right hind leg in all experimental mice (p<0.05) starting from 14 DPI (Figure 2A), reaching up to $5.3 \times 10^2$ spores per 1 g of tissue reported 2 weeks post-inflammation induction. Moreover, the inflammation induction resulted in a significant increase (p<0.05) in concentration of spores in the induced site in the experimental group 35 DPI and prolonged persistence of microsporidia till 56 DPI in the induced leg compared to the control group (Figure 2A).

One order shift towards the induced site was also reported in immunocompetent BALB/c mice infected with E. cuniculi genotype III with induced inflammation, starting at 14 DPI and reaching the maximum of $7.9 \times 10^4$ spores per gram of tissue at 14 DPI. The persistence of microsporidia was prolonged for 2 weeks in the induced right hind leg compared to the control group (Figure 2B).

**Discussion**

Of the several species of microsporidia that infect humans, E. cuniculi belongs to the most common. Horizontally transmitted environmentally resistant life cycle stage spores access host tissues by germinating in the gut lumen, where
infections are typically initiated in gut epithelial cells, and sometimes muscle cells. However, the target tissues — those in which microsporidia develop to the infectious stage, represent a species-specific interaction with its host. As *E. cuniculi* lacks the organ/cell specificity, it can infect a wide spectrum of host cells including epithelial cells, vascular endothelial cells, kidney tubule cells, and possibly other cell types, and can be found in most tissues, with a predilection, in mammals, for the brain and kidney. However, microsporidian spores are non-motile and short distance host dissemination is limited to a unique mechanism of host cell invasion using 10–50 μm long polar tubes that deliver this organism into the host cell. As *Encephalitozoon* species are able to survive and replicate in a variety of immune cells, including resident and migratory macrophages and other phagocytic cells such as neutrophils, and eosinophils, monocytes, and dendritic cells, which may contribute to the dissemination of *E. cuniculi* throughout the host organism, suggesting that induction of chemokines for inducing innate immune inflammation may also promote recruitment of host cells for continued infection and dissemination. Recently, a role for the involvement of such immune cells connected with pro-inflammatory immune responses (eg, trafficking macrophages and other phagocytic cells such as neutrophils, monocytes, dendritic cells, and eosinophils) in the expansion of *E. cuniculi* infection in host tissues has been suggested based on the occurrence of microsporidia in inflamed tissues and the targeted migration seen following experimental induction of inflammation towards inflammation foci.

Although four genotypes of *E. cuniculi* have been identified to date, human infections are mostly associated with *E. cuniculi* genotypes I and II, which are found primarily in HIV-infected patients and in transplant recipients. In animals, *E. cuniculi* genotype III seems to be more aggressive and infections caused by this strain can lead to the death of immunocompetent hosts whereas *E. cuniculi* genotypes I and II mostly represent a parasite that occurs in host species in a latent asymptomatic form.

As the particular genotypes of *E. cuniculi* differ not only in infection onset speed and extent of infection, but also in chemotherapy sensitivity, thus host survival and progression of the infection depend not only on the immune status of the host but also on the *E. cuniculi* genotype causing the infection. Under experimental conditions, the infection extent and albendazole sensitivity of *E. cuniculi* genotype I was comparable to *E. cuniculi* genotype II and the infection onset speed and mortality rate were similar to *E. cuniculi* genotype III.

As the results of the present study show, the migration potential of *E. cuniculi* genotypes and their concentration in induced inflammatory foci varied in both immunocompetent and immunodeficient mice. In SCID mice the concentration of microsporidia was the most intense in *E. cuniculi* genotype III, reaching $1.0\times10^7$ spores per gram of tissue at 35 DPI, compared to $2.4\times10^3$ and $5.0\times10^5$ reported in genotypes I and II, respectively (present study). (Figure 3A). This correspond to the data published by Sak et al and Kotková et al, ie, the infection intensity of *E. cuniculi* genotypes I and II was comparable, whereas *E. cuniculi* genotype III reached up to two orders higher spore burden. Induction of inflammation in the experimental group of SCID mice infected with *E. cuniculi* genotype I or III resulted in a significant increase of spore burden at the site of inflammation, while, in the control group, the amount of spores per gram of tissue was approximately identical in both legs. Moreover, the significant concentration of microsporidia in the inflammation-induced site was comparable in the case of *E. cuniculi* genotypes I and III and was reported from 14 DPI, whereas the only significant difference between induced and non-induced site was described in SCID mice infected *E. cuniculi* genotype II at 21 DPI (Figure 3A). The elevated concentration of spores in muscle tissues at the end of the experiment regardless of inflammation induction could be attributed to the systemic spread of *E. cuniculi* in the terminal phase infection, as described already by Kotková et al.

Also, in control immunocompetent BALB/c mice, the course of infection caused by *E. cuniculi* I was similar to *E. cuniculi* genotype II with a random occurrence of microsporidia in the limbs and infection clearance starting from 42 DPI, compared to *E. cuniculi* genotype III with the infection clearance at 49 DPI (present study), suggesting that, during the experiment, microsporidia may have been eliminated by the host immune system or microsporidia remaining in the body in undetectable amounts. The peak of infection in muscles was reported earlier in *E. cuniculi* genotypes II and III (21 DPI) than in infection with *E. cuniculi* genotype II (28 DPI) (present study).
The induction of inflammation in experimental BALB/c mice led to the disappearance of all genotypes of *E. cuniculi* from the non-induced leg starting either from 14 DPI (genotypes II and III) or 21 DPI (genotype I) (present study), reaching a peak of *E. cuniculi* concentration at 14 DPI (genotype III) or 21 DPI (genotypes I and III). On the other hand, no difference was observed between the occurrence frequencies and spore burden of microsporidia in induced and non-induced legs in the particular control groups (present study).

**Figure 3** Maximum value of *E. cuniculi* spores per 1 gram of tissue (number in white fields), frequency and spore burden of *E. cuniculi* spores per gram of tissue (colored squares; each square represents one mouse), and infection trend illustration (red fields) in immunodeficient SCID mice (A) and immunocompetent BALB/c mice (B) infected with *E. cuniculi* genotype II (modified from Brdičková et al.48). *Peroral infection 10^7* spores of *E. cuniculi* in 200 µL dH₂O and intramuscular injection of PBS; *peroral infection 10^7* spores of *E. cuniculi* in 200 µL dH₂O and intramuscular injection of Freund's Incomplete Adjuvant; grey column, intramuscular injection of all animals in the group into the right thigh muscle; *significant difference between right and left leg; *significant difference between control and experimental group; colored squares, positive capture of microsporidia in the sample according to the quantity scale.

The induction of inflammation in experimental BALB/c mice led to the disappearance of all genotypes of *E. cuniculi* from the non-induced leg starting either from 14 DPI (genotypes II and III) or 21 DPI (genotype I) (present study). Moreover, a significant increase of microsporidia towards the sites of inflammation in the induced leg was observed from 14 DPI in all genotypes, reaching a peak of *E. cuniculi* concentration either at 14 DPI (genotype III) or 21 DPI (genotypes I and III). On the other hand, no difference was observed between the occurrence frequencies and spore burden of microsporidia in induced and non-induced legs in the particular control groups (present study).

**Conclusions**

Based on our results we can conclude that the induction of inflammation had caused increased concentrations of *E. cuniculi* genotypes I and III in inflammatory foci, as previously reported for *E. cuniculi* genotype II. The frequency of microsporidia occurrence was not random, but shifted in favor of the site of induced inflammation compared to control groups, where spores were accidental or their specific DNA was not detected at all.

Moreover, our study confirmed differences among *E. cuniculi* genotypes reported previously based on the infection onset, infection extent, and host mortality rate. Based on the present study, we can conclude that particular genotypes of *E. cuniculi* also differ in dispersal rate within host bodies after experimentally induced inflammation and *E. cuniculi* genotype I possesses a combination of features reported in genotypes II and III.
As the mechanisms of microsporidia dispersal as well as the potential vehicle cells remain speculative, detailed specification is needed. The understanding of the connection of microsporidia dispersal with pro-inflammatory immune responses, addressing the tissue specificity and the mechanisms of transfer to target tissues, represents an important challenge with potential consequences for human health and brings novel insight into microsporidia problems and developing reliable preventive and therapeutic strategies.

**Author Contributions**

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; and have agreed on the journal to which the article has been submitted.

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**Disclosure**

The authors report no conflicts of interest in this work.

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