Přírodovědecká fakulta  Jihočeské univerzity v Českých |Budějovicích

# Protilátková odpověď kuřat infikovaných mikrosporidií *Encephalitozoon hellem*

Rigorózní práce 2014

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**Anotace:**

Immune response against the microsporidium *Encephalitozoon hellem* infection was studied using perorally and intraperitoneally infected two-day-old chicken. The specific antibody response was monitored during two months interval in dropping extracts and blood. While in intraperitoneally infected chicken only IgY was identified in sera, all three izotypes (IgY, IgA, and IgM) were detected in dropping of perorally infected birds.

Prohlašuji, že v souladu s §47b zákona 111/1998 Sb. V platném znění souhlasím se zveřejněním své rigorózní práce, a to v úpravě vzniklé vypuštěním vyznačených částí archivovaných Přírodovědeckou fakultou elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách.

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ORIGINAL PAPER

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# Humoral response of chicken infected with the microsporidium *Encephalitozoon hellem*

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**Abstract** Chicken (*Gallus gallus*) were used as the experimental model for study of immune response against the microsporidium *Encephalitozoon hellem* (Didier et al., J Inf Dis 163:617–621, 1991) infection in birds. Two-day-old chicken were infected perorally or intraperitoneally with a dose of $10^7$ spores of *E. hellem*. The anti-*E. hellem* immunoglobulin (Ig)A, IgY, and IgM antibody responses in sera and dropping sample extracts were determined by enzyme-linked immunosorbent assay. Results have shown specific antibody production in sera and intestinal secretions of infected birds. Chicken inoculated perorally developed the lowest antibody response. Microsporidian spores were not identified in the smears from cloacal swab samples of individual chicken. Intestinal segment cultures of perorally infected chicken cultivated in vitro showed the highest production of specific IgY and IgA antibodies in jejunum segments. In the further course of infection, the colon produced the highest amount of IgA, and the ileum and colon produced the highest amount of IgY.

## Introduction

*Encephalitozoon hellem* is one of the common human microsporidian parasites. This species of the phylum Microspora was first recognized as a human pathogen in the case of three AIDS patients with keratoconjunctivitis (Didier et al. 1991). Additional cases in immunocompromised humans have also been described from the eye, respiratory tract, urogenital tract, and as fatal disseminated systemic infections (Kotler and Orenstein 1998). This parasite can be transferred to *Macaca mulatta* (Didier et al. 1994).

Since the 1980s, diseases associated with microsporidia have been reported in domestic pet birds (*Melopsittacus undulatus*) (Black et al. 1997). In 1998, the first microsporidian infection in nonpsittacine birds was reported in an ostrich (*Struthio camelus*) (Gray et al. 1998). Molecular characterization showed that *E. hellem* is also the cause of microsporidiosis in birds (Snowden and Logan 1999). The identification of *E. hellem* in nonpsittacine birds suggests that microsporidian organisms have a wider avian host range than previously believed.

The sources of human opportunistic microsporidial infection remain speculative. However, possible animal reservoirs of *E. hellem* are emerging. Thus far, humans and rhesus monkeys are the only identified mammalian hosts, although microsporidiosis caused by *E. hellem* is probably common in birds (Black et al. 1997; Pulparampil et al. 1998). However, Concurrent *E. hellem* infection of both humans and their companion birds has not yet been documented (Bryan and Schwartz 1999).

Most species of microsporidia are transmitted by fecal contamination of water and foods. *E. hellem* can enter the host organism through the other mucous membrane, for example, ocular infection (Diesenhouse et al. 1993; Rosberger et al. 1993; Didier et al. 1996; Silverstein et al. 1997). In the case of the birds, dropping is the main source of the *E. hellem* infection of other animals, possibly humans. Therefore, the most frequent way of infection could be the fecal–oral transport. Food or drink contaminated with fecal material and subsequent infection induces a cascade of immunological events that involve both components of the innate and adaptive immunity. Normal defense against ingested pathogens includes (1) acid gastric juice, (2) the viscous mucus layer covering the gut, (3) lytic

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pancreatic enzymes, including bile detergents, and (4) secreted immunoglobulin A (IgA) antibodies. Natural antibodies serve as recognition molecules capable of opsonizing invading microorganisms and initiating the complement enzyme cascade, which ends in cell lysis. Antibodies might play an immunoregulatory role, but the most important physiological role for enhancing survival of the host seems to be early resistance against infection (Ochsenbein and Zinkernagel 2000).

Therefore, the purpose of this study is to verify humoral response of *E. hellem*-infected baby chicken.

## Materials and methods

### Parasites

The spores of *Encephalitozoon hellem* were originally isolated from AIDS patients with keratoconjunctivitis (Didier et al. 1991) and grown in vitro in VERO E6 cells (Green monkey kidney cells) maintained in RPMI-1640 medium (Sigma) supplemented with 2.5% fetal bovine serum (FBS). Spores were isolated, purified from cells by centrifugation in 50% Percoll (Sigma) at 1,100×*g* for 30 min, washed three times in deionized water, and stored in deionized water supplemented with antibiotics (Sigma, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B) at 4°C. The spores were washed in phosphase-buffered saline (PBS) before use.

### Chicken

Baby chicken (*Gallus gallus*) (strain JA 457, ISA HUBBARD, France) were obtained from a commercial hatchery on the day of hatch. The chicken were housed in wire-floored isolation units and provided food and water ad libitum.

### The detection of antibodies

Two-day-old chicken were inoculated perorally (p.o.) (17 chicken) or intraperitoneally (i.p.) (5 chicken) with a dose of $10^7$ spores of *E. hellem*. Negative control group consisted of 17 animals. The anti-*E. hellem* IgY, IgA, and IgM responses in sera and extract of dropping samples were determined by enzyme-linked immunosorbent assay (ELISA) weekly during a 2-month period. Chicken infected i.p. were not tested for production of intestinal antibodies.

The dropping samples were obtained weekly from each p.o.-infected chicken. They were weighed, mixed 1 g:1 ml in PBS containing 1% FBS, centrifuged at 1,100×*g* for 10 min, and the supernatants stored at −20°C until used. Blood samples were taken from the wing vein weekly, and the sera were stored at −20°C until used.

### The detection of spore shedding

Smears made from cloacal swab samples of individual chicken were stained with calcofluor M2R stain and examined using fluorescent microscopy for the presence of spores. The swab samples were taken every day post-p.o. infection during a 2-month period.

### The detection of antibody production in intestinal segments

Every seventh day post- p.o. infection, two chicken were euthanized, and the intestinal segment cultures were performed according to the method of Loa et al. (2002), with modification. Briefly, a 0.5-cm-long segment of the duodenum, jejunum, ileum, cecum, and colon were collected under sterile conditions, opened longitudinally, and washed twice with calcium- and magnesium-free Hanks balanced salt solution (HBSS) supplemented with 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and 50 μg/ml of gentamycin (Lek, Slovenia). Each segment was placed in an individual well of a sterile 24-well culture plate containing 1 ml of RPMI-1640 medium (Sigma) supplemented with 10% normal chicken serum (NCS), antibiotics (Sigma, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin), and 30 μg/ml gentamycin.

### Enzyme-linked immunosorbent essay

The ELISA test was performed according to the method of Hollister and Canning (1987). Briefly, *E. hellem* spores were coated to the plate, incubated with appropriate undiluted dropping supernatants or sera diluted 1:50, and then incubated with 1:5,000 diluted peroxidase-conjugated goat antichicken IgY, IgA, IgM (ICL, Inc., Newberg, OR, USA). The color reaction was developed with 0.1 M acetate substrate solution (pH 5.5) supplemented with 2.5 mM *o*-phenylendiamine (OPD) and 0.03% $H_2O_2$. The reaction was then stopped with 2 M $H_2SO_4$, and the color reaction was measured at 490 nm with an ELISA spectrophotometer (Labsystem Multiscan). Results represent the mean of three samples.

## Results

Chicken inoculated p.o. with *E. hellem* spores were examined for antibody production in both sera and dropping extracts. The i.p.-infected chicken were tested for antibody production in sera only. This group of experimental animals developed stronger humoral immune response than p.o.-infected chicken. Comparison of production of antibody

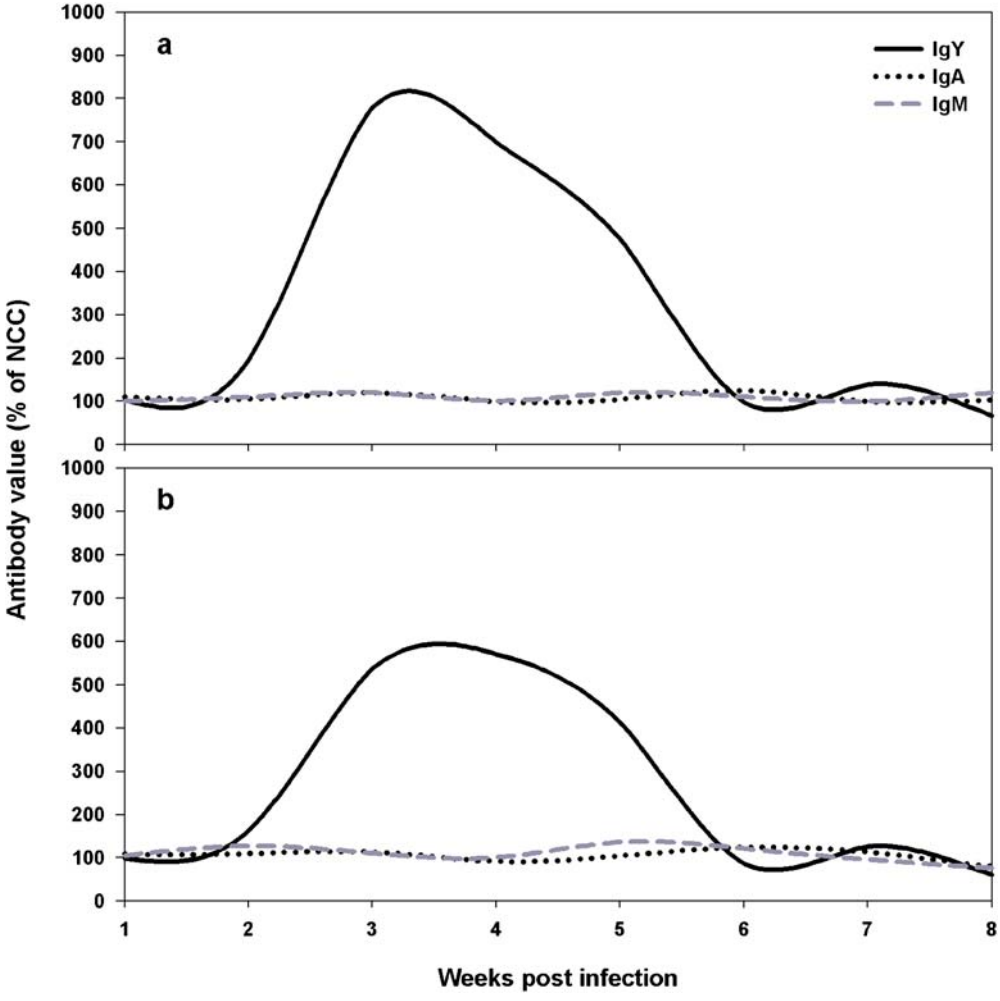


**Fig. 1** Specific antibody concentrations measured in sera of either intraperitoneally (**a**) or perorally (**b**) *E. hellem*-infected chicken (*NCC*, negative control concentration)

isotypes detected in sera of experimental animals is shown in Fig. 1. Only IgY antibody was measured at significant level in both groups (p.o. and i.p.). Intraperitoneally infected chicken produced higher amounts of IgY than p.o.-inoculated animals. IgY increased in the second week postinfection (WPI) and decreased after the fifth WPI. The maximum of IgY production was measured between the third and fourth WPI (779% of NCC) (Fig. 1a). In the case of p.o.-infected chicken, the dynamics of IgY production was similar to that obtained in i.p.-infected chicken, but the total amount of IgY was lower (maximum 570% of NCC on the fourth WPI) (Fig. 1b).

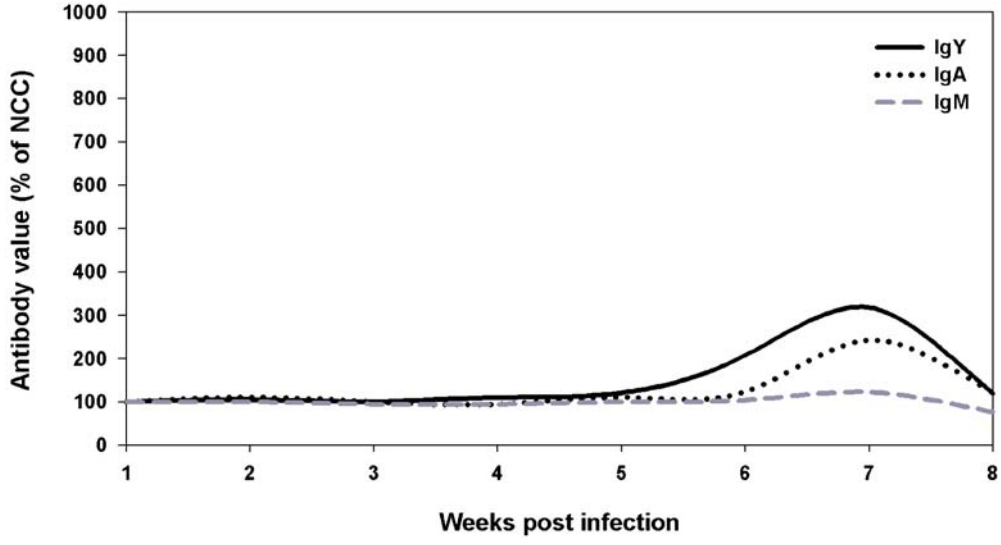


**Fig. 2** Specific antibody concentrations measured in stool extracts of chicken inoculated perorally by *E. hellem* spores (*NCC*, negative control concentration)

The presence of specific antibodies was tested in dropping extracts of p.o.-inoculated chicken only (Fig. 2). Antibody isotypes IgY, IgA, and IgM were detected. The IgY production was stimulated stronger than IgM and IgA antibodies compared with negative control concentration. IgY production increased from the fourth WPI, and the maximum was detected on the seventh WPI (319% of NCC), then the concentration decreased. The production of IgA antibody started to increase from the fifth WPI, and the maximum concentration was obtained on the seventh WPI (244% of NCC). The lowest production was measured in the case of IgM antibody. It increased from the sixth WPI and reached the maximum on the seventh WPI (123% of NCC).

Production of specific IgY and IgA antibodies was observed in intestinal segment cultures of chicken infected p.o. with *E. hellem*. In the first phases of the infection, the major production of IgA and IgY antibodies was measured in segments of the jejunum. In the further course of infection (seventh WPI), the colon produced the highest amount of IgA, and the ileum and colon produced the highest amount of IgY. No IgM production was observed in intestinal segment cultures (data not shown).

Microsporidian spores were not found in the droppings of the chicken sampled using a fluorescent microscopy method. Both p.o.- and i.p.-infected chicken were without any clinical symptoms of microsporidiosis.

## Discussion

Very little is known about humoral response of birds infected with *Encephalitozoon hellem*. Immunocompetent and immunodeficient mice, monkeys, and humans infected with microsporidia develop parasite-specific antibodies (Gannon 1980; Niederkorn and Shadduck 1980; Schmidt and Shadduck 1984; Didier 1995). Chicken IgY antibodies are the equivalent to mammalian IgG antibodies and can be isolated from serum or egg yolk.

The development of antibody responses to *E. hellem* was studied by Snowden et al. (personal communication). Experimentally infected specific pathogen-free (SPF) love birds (*M. undulatus*) seroconverted after 2 WPI. Antibodies were detected by second WPI, and titers were marginally detectable in some birds by 36–38 WPI.

Our results showed that the chicken became infected, but the course of the infection was moderate, and all the experimental animals remained asymptomatic. Antibodies were produced (in blood and in dropping extracts of baby chicken) since the second WPI till the seventh WPI. That could be the effect of species sensitivity, immune response variability, and recovery speed. Our chicken probably recovered from the infection very quickly because the infection was quite moderate. The extensive weight gain of chicken probably caused moderate microsporidiosis and quick recovery. As a result of the mild infection with *E. hellem*, the amount of shedding spores was low and under the limit of detection using fluorescent microscopy.

Intraperitoneally infected chicken produced more antibodies in sera. The cause of this production could be unnatural route of infection, when the inoculated spores did not need to overcome the intestine barriers and could thereby directly get in touch with the immune system.

The course of the localization of mucosal antibody production could reflect the changes in infection localization in time. These changes correlated with antibody values observed in dropping extracts. The decrease of antibodies on the seventh WPI probably reflects the recovery of chicken.

The small size of microsporidian spores and nonspecific staining characteristic make direct detection of spores in feces using fluorescent microscopy complicated. The study of Fayer et al. (2003) showed that suitability of microscopical detection of spores in feces depends on host species, concentration of spores in samples, and species of detected microsporidia. Microscopical detection of *E. hellem* spores in a concentration of 10,000 spores per gram of chicken feces was only 50% positive in comparison with polymerase chain reaction (PCR) detection (Fayer et al. 2003). The *E. hellem* spores in a concentration of 1,000 spores per gram of chicken feces were detectable by PCR method only. Therefore, light microscopy is suitable for basic screening of massive spore-shedding. We could not detect any *E. hellem* spores; however, they could be present in the smears under the detectable limit concentration (at least 10,000 spores per gram of chicken feces). PCR and molecular methods seem to be a more reliable method of detecting spores in droppings of infected animals.

The recent study of Snowden and Phalen (2004) suggests that clinical disease in lovebirds is rare, and the most infections are unapparent. This hypothesis is supported by the present results because none of the infected chicken exhibited any clinical symptoms of microsporidiosis. Our study also showed that whereas no obvious illness was observed, the immune reaction of chicken to microsporidian infection was detected. Despite mild infection and little amount of the spores in droppings (under the limit of detection), such birds could be the source infection for immunodeficient patients and another birds. This hypothesis is supported by Barton et al. (2003), who studied prevalence of microsporidian spores shed in the droppings of apparently healthy lovebirds during a 7-month period. Microsporidian spores were identified in the droppings of 25% of the lovebirds sampled. No difference in spore shedding was found between juvenile and adult birds. The concurrent infection increase probability of spore shedding of asymptomatic birds. Lovebirds concurrently infected with psittacine beak and feather disease virus (PBFDV) were approximately three times more likely to shed microsporidian spores (Barton et al. 2003).

In birds, as in natural hosts, most infections with *E. hellem* remain asymptomatic. *E. hellem* spore-shedding depends on the other concurrent infection. The course of the infection is quite long (several weeks). The droppings of asymptomatic birds infected with *E. hellem* represents a potential source of infection for other birds and for both immunodeficient and immunocompetent humans.

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