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Detection of *Echinococcus multilocularis* antigens in faeces by ELISA

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Abstract Faecal samples deriving from 391 animals belonging to nine species (polecats, badgers, martens, weasels, rats, dogs, cats, red foxes, raccoon-dogs) were examined by capture ELISA for the presence of the *Echinococcus multilocularis* coproantigen. The main claim of our studies is the reliable detection of *E. multilocularis* coproantigens, mainly in the faeces of foxes, dogs and cats. For the first time in coproantigen detection we used a "double-sandwich" ELISA. The main advantage of this method is the higher specificity and better differentiation of positive and negative faecal samples, in comparison with sandwich ELISA. The overall specificity of double-sandwich ELISA was 95.1% with only 16 of 327 *E. multilocularis*-free animals giving false-positive results. The *E. multilocularis* coproantigen was detected by double-sandwich ELISA in 37.5% of examined red foxes and in 8.0% of examined raccoon-dogs, compared with a prevalence of just 29.8% in red foxes and 8.0% in raccoon-dogs, as determined by parasitological techniques.

Introduction

Echinococcus multilocularis is one of the most important zoonotic parasites distributed in the northern hemisphere. Alveolar echinococcosis (AE), caused by larval stages of *E. multilocularis*, is one of the most lethal helmintic infections of humans (Amman and Eckert 1995). Also, cases of *E. multilocularis* in humans have been reported in Poland (Malczewski et al. 1995). The wild fox plays a major role in the parasite life cycle as the main definitive host. The prevalence of *E. multilocularis* in foxes was up to 60% in Central Europe (Eckert and Deplazes 1999). In the northern part of Poland, this parasite infected as much as 36% of the red fox population (Malczewski et al. 1999). It should be taken into consideration that, in parts of Europe, fox population densities have increased from 1985 onwards, following successful oral vaccination campaigns against rabies. This over-population of foxes caused their presence in urban settings and became an important implication for the management, control and prevention of zoonoses (rabies, AE). Foxes and raccoon-dogs penetrating urban communities could be a real threat for domestic pets, by introducing *E. multilocularis* into circulation in a domestic cycle. Domestic dogs and cats have been identified as definitive hosts of *E. multilocularis* (Deplazes et al. 1999; Eckert et al. 1999). The significance of these domestic species for zoonotic transmission needs precise monitoring. Our examinations revealed that the raccoon-dog (*Nyctereutes procyonides*) was the host for *E. multilocularis* throughout Asia and far Eastern Europe and also in Eastern Germany (Machnicka-Rowinska et al. 2001, 2002; Thiess et al. 2001).

The standard post-mortem diagnosis of *E. multilocularis* in final hosts (fox, dog, cat) is the sedimentation and counting technique (Eckert et al. 1999). This method also allows determining the stages of development of the intestinal worms and precise quantification of the parasite biomass. At present, parasitological examination of the small intestine at necropsy with the intestinal scraping technique is the standard used in epidemiological investigations (Deplazes and Eckert 1996). However, these parasitological methods are very laborious, special safety precautions must be taken and this kind of examination can be applied to dead animals only. Immunological methods based on the detection of *E. multilocularis*-specific coproantigens have been applied in recent years to diagnose *E. multilocularis* infections. The detection of coproantigen could be very useful for field surveys of suspected hosts naturally infected with *E. multilocularis*, because of its simplicity, safety and relatively high sensitivity (Eckert et al. 1984). This

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kind of examination could replace necropsy. Infection with adult cestodes could also be demonstrated by the detection in faecal samples of parasite-specific DNA fragments originating either from parasite eggs or from the cells or tissue debris of tapeworms (Gottstein and Mowatt 1991; Bretagne et al. 1993; Dinkel et al. 1998). The high costs connected with a DNA-identification method exclude it from epidemiological examinations.

The aim of our study was to apply a modified capture ELISA method to detect specific *E. multilocularis* coproantigens in wild and domestic animals deriving from different regions of Poland and to compare them with the results of parasitological examinations. Special attention was paid to dogs and cats, which could became hosts of *E. multilocularis*.

Materials and methods

In our study, we examined 391 animals belonging to nine species: two polecats (*Mustela putorius*), two badgers (*Meles meles*), two martens (*Martes* sp.), one weasel (*Mustela nivalis*), one rat (*Rattus* sp.), 110 dogs (*Canis familiaris*), 40 cats (*Felis catus*), 208 red foxes (*Vulpes vulpes*) and 25 raccoon-dogs (*Nyctereutes procyonoides*). The alimentary tracts of these animals were examined for the presence of helminths. The exception was 53 red foxes, which were examined only to detect *E. multilocularis*. Of the specimens examined, 216 animals came from the northern part of Poland and 175 animals from the southern part of Poland. In order to exclude any infection risk for laboratory personnel, the carcasses or intestines were frozen at −80 °C for at least 10 days before examination (Deplazes et al. 1992, 1999).

Parasitological examination

The alimentary canal of each animals was isolated and examined for the presence of *E. multilocularis*, using the scraping technique. In brief, the small intestines were thawed and each was cut into five equal parts. Each part was then cut longitudinally and, after removing the contents, three mucosal scrapings were taken for microscopic slide examination (Eckert et al. 1984). Worm burdens were determined as low (<100 *E. multilocularis*), medium (100–1,000) and high ($>1{,}000$ parasites).

Detection of specific *E. multilocularis* coproantigens

Faecal sample preparation

Four different dilution buffers were compared: (1) Phosphate-buffered saline (PBS), (2) PBS and 0.3% Tween-20, (3) PBS, 0.3% Tween-20, 0.05% bovine haemoglobin and 0.04% NaN_3 and (4) Citric buffer (pH 5.0) and 5% bovine serum albumin (BSA). The weighed faecal samples were mixed with dilution buffer at a ratio of 1:5, shaken and centrifuged at 6,000 *g* for 10 min and then the supernatants were frozen at −20 °C until further processed. Before examination, the faecal supernatants were thawed, mixed with the dilution buffer at a ratio of 1:2 and centrifuged at 4 °C at 12,000 *g* for 30 min and then the supernatant fluid was directly used in ELISA.

Preparation of E. multilocularis antigens

Adult *E. multilocularis* tapeworms were recovered from the intestines of naturally infected red foxes. After several washings with sterile 0.85% NaCl, the parasites were homogenised, ultrasonicated with sterile PBS and centrifuged at 0 °C for 30 min at 10,000 *g*. Protein determination was done by the method of Sedmak and Grossberg (1977) with Coomassie Blue. After appropriate dilution, the supernatant was used as a somatic antigen.

Production of polyclonal antibodies

Polyclonal antibodies were raised in rabbits by a single intralymph-node injection of 100 μg of somatic *E. multilocularis* antigens followed by repeated subcutaneous injections of 100 μg of somatic *E. multilocularis* antigens (alternately injections of complete or incomplete Freund's adjuvant) at weekly intervals. Immunoglobulins (Rab) were precipitated by ammonium sulphate (Hudson and Hay 1980) from immunised rabbit's sera.

Conjugate preparation

The peroxidase conjugation of rabbit's anti-*E. multilocularis* immunoglobulins (RabP) was carried out according to the technique described by Nilson and Nakane (1978). The conjugated antibodies were separated on a Sephadex G-200 column. Collected fractions were monitored at 280 nm and 403 nm in a Beckman spectrophotometer; and those showing parallel peaks in both lengths were selected for use after concentration.

Enzyme-linked immunosorbent assay

Optimal dilution of reagents was determined by checker-board titration. Some of the faecal samples (67) were examined by both sandwich ELISA and double-sandwich ELISA in parallel. Next, all faecal samples were examined by double-sandwich ELISA only.

Sandwich ELISA

Flat-bottomed microtitre plates (Nunc, Maxisorp) were coated overnight at 4 °C with 200 μl of Rab containing 20 μg of protein/ml in 0.005 *M* carbonate buffer (pH 9.6) per well. After washing three times with PBS containing 0.05% Tween-20 (PBST), 200 μl of the duplicate faecal samples (diluted at 1:10 in dilution buffer 1, 2, 3 or 4) were added and the plates were incubated at 37 °C for 1 h. After washing, 200 μl of RabP per well (diluted 1:50 in PBST containing 0.5% BSA; PBSTA) was added and incubated for 1 h at 37 °C. After washing, 100 μl of substrate was added: *o*-phenylenediamine (Sigma) in 0.1 *M* phospho-citric buffer (pH 5.0) with 0.04% hydrogen peroxide. The reaction was stopped with 4 *N* sulphuric acid and the plates were read using an automatic 96-well reader (Organon Teknika) at 492 nm.

Double-sandwich ELISA

The plates were coated with somatic antigens of *E. multilocularis* containing 2 μg of protein coating buffer/ml and incubated overnight at 4 °C. After washing, Rab containing 20 μg protein/ml diluted in PBSTA was added and incubated at 37 °C for 1 h. Next, the faecal samples, diluted as above, were added and incubated at 37 °C for 1 h. Further procedures were the same as in sandwich ELISA.

Faecal samples from red foxes not infected with *E. multilocularis* were processed as described above and used as negative controls. Control negative and positive samples were included in each assay.

The double-sandwich assay was introduced to improve the specificity of the method. The antigen-specific antibody of the second level were bound to the specific epitopes of the coated antigen. The excess and unbound antibody was washed out from the plate. The antigens from examined samples (if present) were

bound to antigen-specific antibody. The conjugated antibodies could bind only to the coproantigen in faecal samples (the specific epitopes of coated somatic antigens were bound to the antigen-specific antibody from the second level). The peroxidase-linked indicator antibodies were measured by spectrophotometer.

Evaluation of results

The discrimination of negative and positive results of ELISA was made by calculating the index value of each examined faecal sample, dividing its optical density (OD) by the OD of the negative control sample. To calculate the cut-off value, the panel of negative faecal samples was tested and the mean value of its OD plus one standard deviation gave an index of 1.5. Values greater than 1.5 were considered positive.

The specificity of the coproantigen-detection test was assessed by the percentage of coproantigen-positive cases found in worm detection-positive cases.

Results

Results of parasitological examinations

The parasites found after necropsy of 338 animals were: *Mezocestaides lineatus* in 95 red foxes (61.2%) and 11 raccoon dogs (44.0%), *Taenia* spp. in 18 red foxes (11.6%), 3 raccoon dogs (12.0%), 1 dog (0.9%), 8 cats(20.0%), 1 marten and 1 badger, *Dipylidium caninum* in 6 dogs (5.4%) and 1 cat (2.5%), *Alaria alata* in 16 foxes (10.3%) and 11 raccoon-dogs (44.0%), *Echinochasmus perfoliatus* in 1 raccoon-dog (4.0%), *Toxocara* spp. in 42 foxes (27.0%), 8 raccoon-dogs (32.0%), 23 dogs (20.9%) and 12 cats (30.0%), *Uncinaria stenocephala* in 44 foxes (28.3%), 8 raccoon-dogs (32.0%), 3 dogs (2.7%) and 2 cats (5.0%) and *Echinococcus multilocularis* in 62 red foxes (29.8% out of 208) and 2 raccoon-dogs (8.0%).

Results of ELISA

The comparison of four dilution buffers showed that the buffer containing 0.3% Tween 20, 0.05% bovine haemoglobin and 0.04% NaN_3 appeared the most suitable for yielding the best discrimination between the results of negative and positive control samples (data not shown).

Altogether, 391 faecal samples were examined by ELISA. As the first step, 67 faecal samples were examined by both sandwich and double-sandwich ELISA in parallel. The examination was made on 22 faecal samples from red foxes infected with *E. multilocularis* (17 derived from foxes in which only the presence of *E. multilocularis* was registered during autopsy, five from foxes in which both *E. multilocularis* and the other helminths were detected and determined) and on 45 faecal samples from red foxes not infected with *E. multilocularis*, as stated at autopsy. The results of these comparative examinations pointed out a higher number of reliable results in double-sandwich ELISA than in sandwich ELISA (Table 1). What is more, a statistically higher absorbance of positive samples was registered in double-sandwich ELISA than in sandwich ELISA (Table 2).

In consequence, after comparing the results of sandwich and double-sandwich ELISA, all 391 faecal samples were examined by double-sandwich ELISA only.

E. multilocularis coproantigens were found in 78 faecal samples from red foxes and in two faecal samples from raccoon-dogs (Table 3).

One false negative result was found in faecal samples from a red fox infected with both *E. multilocularis* and *Taenia* spp., *Mezocestaides lineatus* and *Uncinaria*.

A total of 16 false positive results were found in faecal samples from red foxes infected with: *Taenia* spp and + *Toxocara* (two foxes), *Mezocestaides* and *Toxocara* (four foxes), *Mezocestaides* (five foxes), *Mezocestaides* and *Uncinaria* (one fox), *Uncinaria* (one fox), *Toxocara* (one fox), *Taenia* spp, *Mezocestaides* and *Toxocara* (one fox) and *Taenia* spp, *Mezocestaides*, *Toxocara* and *Uncinaria* (one fox).

Overall, the coproantigen prevalence was 37.5% in red foxes and 8.0% in raccoon-dogs, as compared with a prevalence of 29.8% in red foxes and 8.0% in raccoon-dogs, as determined by the parasitological technique.

Table 1 Comparison of the results of sandwich ELISA with double-sandwich ELISA. Examinations were done on *Echinococcus multilocularis* positive and negative faecal samples

Parasitological examination after necropsy	Results of sandwich ELISA	Results of double-sandwich ELISA
22 positive	13 positive	27 positive
–	Mean absorbance = 0.29	Mean absorbance = 0.41
–	4 uncertain	1 uncertain
45 negative	50 negative	39 negative

Table 2 Comparison of mean absorbance obtained in sandwich ELISA and double-sandwich ELISA

	Sandwich ELISA	Double-sandwich ELISA
Mean absorbance of positive examined samples	0.29	0.41
Mean absorbance of control positive samples	0.19	0.25
Mean absorbance of control negative samples	0.12	0.09
Cut-off	0.18	0.13

Table 3 The results of examinations by double-sandwich ELISA of faecal samples of red foxes, raccoon dogs, domestic dogs and cats in comparison with positive or negative *E. multilocularis* detection

Number of examined samples	*E. multilocularis* stated after necropsy	Coproantigen positive by ELISA	False positive in coproantigen ELISA	False negative in coproantigen ELISA
208 red foxes	62	78	16 (7.6%)	1 (1.6%)
25 raccoon dogs	2	2	0	0
110 dogs	0	0	0	0
40 cats	0	0	0	0
All examined ($n = 391$)	64	80	16 (4.0%)	1 (1.5%)

The overall specificity of double-sandwich ELISA was 95, 1.0% with only 16 of 327 *E. multilocularis*-free animals giving false-positive results.

Results of parasitological and ELISA examinations

Only two of nine examined species occurred as a reservoir of *E. multilocularis*. Dogs and cats, although originating from a region of high prevalence of *E. multilocularis* in red foxes, were free of infection with this parasite. The samples of these animals showed no non-specific positive results, while they were infected with different parasites (*Taenia* spp in one dog, eight cats; *Dipylidium caninum* in six dogs, one cat; *Toxocara* spp in 23 dogs, 12 cats; *Uncinaria stenocephala* in three dogs, two cats).

The present study revealed that both the red fox and the raccoon-dog are a wild reservoir of *E. multilocularis* in Poland.

Discussion

It was shown by Ballek (1991) that the sensitivity of parasitological examination of foxes for the presence of *Echinococcus multilocularis* at necropsy is about 96.0%. In considering losses due to autolysis processes, an estimated sensitivity of 85.0% may be realistic. Hence, there is a need to elaborate a detection method which is sensitive and safe for researchers.

Several researchers have described ELISAs for the detection of coproantigens released by *E. multilocularis*. Previous studies (Deplazes et al. 1990) showed that *Taenia* spp and *Echinococcus* spp coproantigens were detectable in faecal samples of infected hosts and remained stable for at least 5 days if the samples were maintained at room temperature. According to Sakai (1998a), the coproantigens could be detected regardless of faecal condition; and thus ELISA is useful even on faeces excreted more than 1 week previously in the field. Coproantigens are detectable during the prepatent and patent periods. They disappear within a few days after the elimination of the cestodes from the host; and antigen concentrations appear to be correlated to the worm burden. Deplazes et al. (1992) stated that the diagnostic sensitivity of coproantigen ELISA was closely dependent on the *Echinococcus* worm burden in natural and experimental infections. The overall diagnostic sensitivity of the coproantigen test according to Deplazes et al. (1999) was 83.6% in foxes infected with *E. multilocularis*; and it reached 93.3% in foxes harbouring more than 20 worms but dropped to 40.0% in animals with worm burdens less than 20. An epidemiological study carried out on wild red foxes in Switzerland showed that 67.1% of the infected foxes had less than 100 *E. multilocularis* (Ewald and Eckert 1993). Similar observations derive from our studies: a low parasite burden (less than 100 worms) was found in 43 (69.3%) infected foxes, 12 (19.3%) had over 1,000 worms and seven (11.2%) had 100–1,000 *E. multilocularis*. Also, Deplazes et al. (1994) stated that the diagnostic sensitivity in stray dogs infected with *E. granulosus* was in close association to the worm burden of infected individuals: when dogs harboured less than 100 worms, 29.0% were found to be positive in coproantigen ELISA, whereas the sensitivity was much higher (92.0%) when dogs had more than 100 worms. According to Sakai et al. (1998b), the coproantigens could not be detected by ELISA in samples from animals harbouring quite a small number of worms. The reliability of the result could not be evaluated, since the true infection status of worm-negative foxes was not determined The detection limit of around 100 worms in experimentally infected foxes was estimated by Nonaka et al. (1996), who applied antibodies against *E. multilocularis* excretory/secretory (ES) antigens in sandwich ELISA. Also, monoclonal antibodies against ES antigens revealed coproantigens as early as 3–5 days post-infection; and the limit of detection was 4 ng of *E. multilocularis* antigen in 1 g of faeces (Sakashita et al. 1995). Deplazes et al. (1992) pointed out that, in the field, the irregular and different feeding habits of wild animals may influence and reduce the stability of coproantigens and subsequently affect the detection limit of the assay.

Our study differed in two ways from previous reports on *E. multilocularis* coproantigen detection. First, the double-sandwich ELISA was performed with somatic *E. multilocularis* antigens as the first layer and the anti-*E. multilocularis* antibodies were achieved by the immunisation of rabbits with the complete adult antigen. The specificity of coproantigen detection in the double-sandwich ELISA was thus improved by the selection of specific antibodies (in the polyclonal immunoglobulin) by parasite antigens. Second, our studies were carried out on the nine species of wild and

domestic animals naturally infected with various intestinal helminths, which gives possibilities for the detection of coproantigen cross-reactivity.

A total of 182 of 208 red foxes (87.5%) and 18 of 25 raccoon-dogs (72.0%) examined after necropsy carried intestinal parasites. The highest prevalence in red foxes (61.2%) was recorded for *Mezocestaides*, followed by *Uncinaria* (28.3%) and *Toxocara* (27.0%). The highest prevalence in raccoon-dogs (44.0%) was recorded for *Alaria*, followed by *Mezocestaides*, *Toxocara* and *Uncinaria* (32.0%). *Taenia* spp were found in red foxes and raccoon-dogs in 5.1% and 12%, respectively.

For the first time in coproantigen detection, we used double-sandwich ELISA. The main advantage of this method was a better differentiation between positive and negative faecal samples (Table 1), in comparison with sandwich ELISA. The diagnostic sensitivity of the double-sandwich ELISA was determined by associating ELISA results to those of intestinal necropsy examination. In our study, the specificity of the double-sandwich ELISA was found to be 98.3%, if 16 positive ELISA results that could not be confirmed by other methods were classified as false-positive reactions. The high specificity of the described double-sandwich was demonstrated primarily by the fact that a wide spectrum of antigens derived from non-echinococcal cestodes or other helminths did not induce significant levels of cross-reactivity. The specificity of the double-sandwich ELISA was determined in surveys of wild foxes and raccoon-dogs and domestic dog and cat populations.

Deplazes et al. (1999) stated specific coproantigen reactions in 0.76% of examined faecal samples from dogs and in 0.76% of faecal samples from cats. The close presence of foxes to human habitats creates the conditions for the infection of domestic dogs and cats with *E. multilocularis*. The proof achieved in our examinations that the faeces of cats and dogs (not infected with *E. multilocularis*) gave negative results in ELISA was of great value. Dogs and cats as a potential source of human infection with *E. multilocularis* create a real threat. The results of experimental studies revealed that cats appear to be less susceptible to *E. multilocularis* than dogs, with a retarded parasite development and lower worm burdens (Crellin et al. 1981; Thompson and Eckert 1983; Kamiya et al. 1985, 1986). However, in the study by Deplazes et al. (1999) three of the five infected young cats harboured more than 1,000 worms.

The very high negative predictive values of the double-sandwich ELISA indicate that the use of this test is especially suited for the mass-screening of a definitive host population with a low prevalence of *E. multilocularis*.

The main problem in coproantigen detection by ELISA is the false-positive and false-negative results, compared with parasitological results at necropsy. The false-negative results can be connected with the presence of a small number of parasites. In our studies, the false-positive results occurred in 16 samples (7.6%) of *E. multilocularis*-negative foxes. Further analysis of the parasite genera found at necropsy did not point out any species responsible for the false serology. What is more, faecal samples deriving from dogs and cats, which had different parasites, often the same as foxes, but were not infected with *E. multilocularis*, showed only negative results in ELISA. In our opinion, the condition of the faecal samples could influence the results of ELISA. The faecal samples of dogs and cats were frozen freshly after euthanasia, while the digestive tracts of foxes derived from hunting could be stored in unsuitable conditions before collection in the veterinary laboratory. From our examination, it appears that the raccoon-dog (being the important wild reservoir of rabies in Poland) should also be monitored as the host of *E. multilocularis*.

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