

A comparative study of different immunoassays to detect specific antibodies to *Echinococcus* spp. in human sera

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Summary

Human echinococcosis, one of the most serious of parasitic zoonoses, is caused by the larval stages of taeniid cestodes of the genus *Echinococcus*. The study aimed to assess the reliability of the detection of specific antibodies to *E. multilocularis* and *E. granulosus* s.l. in human sera and to compare their diagnostic potential for their utilization in the practice. In the study, the somatic antigen of *E. multilocularis* (AgEm), antigen B (AgB), and the hydatid fluid antigen of *E. granulosus* and two commercial ELISA kits – *Echinococcus granulosus* (Bordier Affinity Products, Crissier, Switzerland) and NovaLisa™ *Echinococcus* IgG (NovaTec Immunodiagnostica, Germany) – were compared. Sera of patients with alveolar and cystic echinococcosis, and with different parasitic/other infections were used to evaluate the sensitivity, specificity and cross-reactivity of in-house and commercial ELISA methods. AgEm presented the highest values regarding the diagnostic indicators, showing 100 % specificity and 90.0 % sensitivity. The tests for serological diagnostics of cystic echinococcosis were less sensitive and specific. The *Echinococcus granulosus* kit had 83.8 % specificity and 88.2 % sensitivity, while AgB and AgHF showed 85.0 % and 86.3 % specificity, and 76.5 % and 100 % sensitivity, respectively. NovaLisa™ *Echinococcus* IgG proved to have 95.7 % specificity and 77.8 % sensitivity. The results point out that the combination of different serological tests and approaches in accordance with clinical and imaging findings is still essential to prove the correct diagnosis in suspected patients.

Keywords: *Echinococcus multilocularis*; *Echinococcus granulosus*; Echinococcosis; ELISA; sensitivity; specificity

Introduction

Human echinococcosis, one of the most serious of parasitic zoonoses, is caused by the larval stages of taeniid cestodes of the genus *Echinococcus*. The species of major medical and public health importance of this genus are *Echinococcus multilocularis* and *E. granulosus* sensu lato (s.l.) the causative agents of alveolar (AE) and cystic echinococcosis (CE), respectively. Other species of public health concern, *Echinococcus vogeli* and *Echi-*

nococcus oligarthus, are responsible for polycystic echinococcosis in Central and South America.

E. multilocularis and *E. granulosus* s.l. are known to circulate on Slovakia territory. *E. multilocularis* has been identified throughout the country, with high-endemic areas in northern districts of the Prešov, Trenčín and Žilina Regions, where the prevalence rates of 39.1 % – 49.6 % were found (Miterpáková & Dubinský, 2011). Examination of cystic material from pigs, cattle and human patients by Šnábel *et al.* (2016) showed the *E. canadensis*, formerly

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the G7 (pig) strain of *E. granulosus* s.l., in the country is present. Moreover, new cases of human AE and CE are reported every year (Antolová *et al.*, 2014; Antolová *et al.*, 2019).

Without a careful clinical management, both AE and CE have a poor prognosis and can result in the death of an infected patient. The diagnosis of infection should be based on clinical and laboratory findings. Since AE and CE have no pathognomonic clinical signs, their diagnosis relies mainly on the results of imaging methods and serological examinations. To confirm the disease, a histopathological examination or detection of parasite-specific DNA from cystic material can also be applied.

Enzyme-linked immunosorbent assay (ELISA) is the most widely used method to evaluate the presence of antibodies to *Echinococcus* spp. The disadvantages are the cross reactivity between *E. multilocularis* and *E. granulosus* s.l. due to some common surface antigens, cross-reactivity with other parasitic species and the absence of antibody production in approximately about of 5 % of patients (Eckert & Deplazes, 2004). The literature often contains contradictory reports regarding the efficiency and accuracy of serological assays, suggesting that their efficacy basically depends on the antigen used (Schweiger *et al.*, 2012). Therefore, studies on the sensitivity and specificity of *Echinococcus* spp. antigens and serological tests are of particular importance and provide relevant information for differential diagnosis of infection in patients suspected of having alveolar or cystic echinococcosis.

Within the study, different *Echinococcus* spp. antigens used in in-house ELISA and commercial ELISA kits were tested to assess the reliability of detection of species-specific antibodies to *E. multilocularis* and *E. granulosus* s.l. in human sera and to compare their diagnostic potential for use in medical diagnostic practice.

Material and Methods

Sample collection

Sera of ten patients with alveolar echinococcosis (AE) and seventeen samples from patients with cystic echinococcosis (CE), obtained in cooperation with Clinics of Infectology in University Hospital Martin in Martin and University Hospital L. Pasteur in Košice, were used in the evaluation of sensitivity (Se) of antigens and commercial ELISA kits (Table 1, 2). The diagnosis of AE/CE was confirmed by the results of positive species-specific serological tests, and by the presence of characteristic imaging findings as well as on histopathology results and/or molecular examinations. Sera of patients with different parasitic/other infections (n = 45), namely ascariasis (n = 8), trichuriasis (n = 4), trichinellosis (n = 8), toxocariasis (n = 9), toxoplasmosis (n = 8), strongyloidiasis (n = 6), dirofilariasis (n = 1) and rickettsiosis (n = 1), and sera from clinically healthy persons (n = 25) were used for cross-reactivity studies (Table 2), and for the evaluation of the antigen specificity (Sp) of in-house as well as commercial ELISA methods (Table 3). The above-mentioned diseases were diagnosed based on the results of parasitological (coprological) examination (ascariasis, trichuria-

sis and strongyloidiasis), a combination of clinical and serological examinations (toxocariasis, trichinellosis and toxoplasmosis) and the results of clinical findings and molecular analyses (dirofilariasis and rickettsiosis). The study was in accordance with the 1975 Declaration of Helsinki, as revised in 2013, and approved by the Ethics Committee of Institute of Parasitology of SAS (No. EK 04/2015).

Antigens

The somatic antigen of *E. multilocularis* (AgEm) (Turčeková *et al.*, 2004) was used to determine the presence of antibodies to *E. multilocularis*. Antibodies to *E. granulosus* s.l. were detected using antigen B (AgB) prepared according to Reiterová *et al.* (2014) and crude antigens of sheep hydatid fluid (AgHF) prepared according to Turčeková *et al.* (2004), with some modifications. Optimal dilutions for each antigen were based on the results of previous titrations, and the final protein concentration was 2.5 µg.ml⁻¹ for antigens AgEm and AgB, and 5.0 µg.ml⁻¹ for AgHF.

Commercial ELISA methods

Two different ELISA methods were included in the study – *Echinococcus granulosus* (Bordier Affinity Products, Crissier, Switzerland) sensitized with hydatid fluid antigens from *E. granulosus* s.l. and NovaLisa™ *Echinococcus* IgG (NovaTec Immunodiagnostica, Germany) coated with *Echinococcus* crude antigen.

Serological examination

ELISA tests with the somatic antigen of *E. multilocularis*, antigen B and hydatid fluid of *E. granulosus* s.l. were performed according to the method modified by Havasiová-Reiterová *et al.* (1995).

Microtiter plates (Nunc; Maxisorp, Denmark) were coated with antigen diluted in carbonate buffer (pH 9.6) overnight at 4°C. Afterwards, the plates were washed three times with distilled water containing 0.05 % Tween-20 (washing solution) and 100 µl of sera diluted 1:200 in 5 % non-fat milk in a phosphate buffer containing 0.05 % Tween-20 (PBS-MT; pH 7.2) were filled into the wells. After 1 hour of incubation at 37 °C, the plates were washed as described previously.

The next steps was addition of 100 µl of IgG peroxidase-labelled conjugate (Goat Anti-Human IgG, Sigma-Aldrich, Missouri, USA) diluted at 1: 30,000 in PBS-MT, followed by 1 hour incubation at 37 °C and subsequent washing. The substrate, 100 µl of o-phenylenediamine/methanol/PBS with 0.05 % H₂O₂, was finally added to visualize the antibody response. The reaction was stopped after 20 min of incubation in the dark at room temperature using 50 µl of 4N H₂SO₄, and optical densities were read spectrophotometrically at 490 nm (OD₄₉₀).

The cut-off values for each tested antigen were based on the results of previous titrations where the sera from 40 blood donors without clinical signs of any disease were tested. The average optical density (OD₄₉₀) plus four standard deviations (SD) was determined as the cut-off. Commercial ELISA tests were performed and evaluated according to the manufacturer's instructions.

Table 1. Correct seropositive reactions in patients with confirmed alveolar or cystic echinococcosis.

Parasitic disease	Antigens/ELISA tests used				
	<i>E. multilocularis</i>		<i>E. granulosus</i> s.l.		<i>Echinococcus</i> spp.
	AgEm	AgB	AgHF	<i>Echinococcus granulosus</i> (Bordier)	NovaLisa™ <i>Echinococcus</i> IgG (NovaTec)
Alveolar echinococcosis (n = 10)	9	n.c.	n.c.	n.c.	8
Cystic echinococcosis (n = 17)	n.c.	13	17	15	13
Total correctly positive/tested	9/10	13/17	17/17	15/17	21/27
Sensitivity (%)	90.0	76.5	100	88.2	77.8

AgEm – somatic antigen of *E. multilocularis*; AgB – antigen B of *E. granulosus*; AgHF – hydatid fluid antigen of *E. granulosus*; n.c. – not calculated

Data and statistical analysis

The cross-reactions between AE and CE and with serum antibodies of patients with different parasitic/other infections were evaluated for each antigen/test.

The individual test indicators, namely sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV) and the Youden index (YI), were calculated to assess the reliability of the test results.

The sensitivity (Se) was determined by dividing the number of true seropositive (TsP) samples by the total number of false seronegative samples (FsN) and TsP:

$$Se = TsP / (FsN + TsP).$$

The specificity (Sp) was expressed by dividing the number of true seronegative samples (TsN) by the total number of false seropositive (FsP) and TsN (Kováč, 1994):

$$Sp = TsN / (FsP + TsN).$$

The positive predictive value indicates the probability that the disease is present when the test is positive, and the negative predictive value suggests the proportion that is without the disease when the test is negative (Altman & Bland, 1994):

$$PPV = \frac{Se \times prevalence}{Se \times prevalence + (1 - Sp) \times (1 - prevalence)}$$

$$NPV = \frac{Sp \times (1 - prevalence)}{(1 - Se) \times prevalence + Sp \times (1 - prevalence)}$$

To summarize the performance of diagnostic tests, the Youden index (YI) was calculated.

Its value ranges from 0 through 1, with a zero value indicating that a test gives the same proportion of positive results for groups with and without the disease, i.e. the test is useless, and a value of 1 indicating that there are no false positives or false negatives, and the test is perfect (Greiner *et al.*, 1995):

$$YI = Se + Sp - 1$$

Exact binomial 95 % confidence intervals (95 % CI) for means of binominal variables were calculated with unweighted data.

Results

Cross-reactivity and specificity of tested antigens and ELISA methods

Cross-reactivity between AE and CE and with antibodies in sera of 45 patients with different parasitic/other infections and 25 healthy persons were evaluated for each antigen/test (Table 1, 2). Among the compared antigens/tests, the AgEm gave no false positive reaction, indicating its 100 % specificity (Table 3).

When antigens and tests intended for the diagnosis of cystic echinococcosis were evaluated, the most common cross-reactions occurred with sera of patients with AE. The commercial *Echinococcus granulosus* (Bordier Affinity Products, Crissier, Switzerland) test gave 10 false-positive results (83.8 % specificity), while ELISA with AgB and AgHF antigens showed 9 and 7 false-positive results, respectively. Cross-reactions with sera of patients with strongyloidiasis also appeared – two samples were positive for antigen B and one for the *Echinococcus granulosus* commercial test (Bordier Affinity Products, Crissier, Switzerland) (Table 2, 3). NovaLisa™ *Echinococcus* IgG (NovaTec Immunodiagnostica, Germany) cross-reacted with the serum of one trichinellosis patient, and with two sera of clinically healthy persons, thus achieving specificity of 95.7 %

Sensitivity, positive predictive values and negative predictive values of tested antigens and ELISA tests

AgEm detected 9 of 10 sera from AE patients, thus showing 90 % sensitivity. The positive and negative predictive values of AgEm also reached high rates, 100 % and 98.9 % (Table 3).

Antigen B (AgB), antigens of hydatid fluid of *E. granulosus* s.l. (AgHF) and the *Echinococcus granulosus* commercial test (Bordier Affinity Products, Crissier, Switzerland) were tested to evaluate the reliability of the detection of specific antibodies to *E. granulosus* s.l. Their sensitivities varied from 76.5 % for AgB, 88.2 % for *Echinococcus granulosus* (Bordier Affinity Products, Crissier, Switzerland) and 100 % for AgHF. In all three tests, the positive predictive

values were lower (52 %, 53.6 % and 60.7 %) than negative predictive values (94.4 %, 97.1 % and 100 %).

NovaLisa™ *Echinococcus* IgG (NovaTec Immunodiagnostica, Germany), which detects IgG antibodies to *Echinococcus* spp., showed lower sensitivity (77.8 %), and the PPV and NPV were 87.5 % and 91.8 %, respectively.

Efficiency and accuracy of tests

The values of the Youden index of all tested antigens/tests are compared in Table 3. AgEm had the highest positive value (0.90) and seems to be the most appropriate tool for serological diagnosis of AE.

AgHF with a Youden index of 0.86, showed to be the most appropriate for the detection of antibodies to *E. granulosus* s.l. in human sera.

Discussion

Human echinococcosis is a serious parasitic disease that regularly occurs in Slovakia. Direct diagnosis of echinococcosis is possible only after the sampling of cystic material through a more or less invasive approaches (biopsy, puncture, surgery), what is associated with the risk of parasite dissemination to the other organs and tissues. Thus, confirmation of the diagnosis in suspected patients primarily depends on the results of imaging methods and serological examinations (McManus *et al.*, 2003). The use of highly sensitive and specific antigens for serological diagnosis of the disease is therefore an essential part of the diagnostic process and ultimately affects the success of treatment and prognosis of the patient. However, the efforts to make species-specific antigens

that are easy to produce and applicable in routine practice is connected with many difficulties. Moreover, the intensity of the serological response varies considerably, depending on the antigen quality, assay methodology and the location and character of the parasitic cysts (Brunetti *et al.*, 2010). Therefore, the present study tested serological methods for assessment the reliability of the results and comparison of their diagnostic potential to determine the presence of species-specific antibodies to *E. multilocularis* and *E. granulosus* s.l. in human sera.

Different antigens and their combinations are used for the serological diagnosis of alveolar and cystic echinococcosis. The diagnosis of alveolar echinococcosis is commonly based on the somatic antigen of *E. multilocularis* (AgEm). In our study, the somatic antigen of *E. multilocularis* (AgEm) showed sensitivity of 90 % and specificity of 100 %, with the same rate of positive predictive value (100 %), and 98.9 % negative predictive value. Although in the study of Reiterová *et al.* (2014) ELISA with AgEm showed cross-reactivity with CE in 91.3 % of cases. In our study AgEm did not identify as positive any of the 17 sera of patients with CE. This could be due to the differences in the procedure of antigen preparation or by differences in the quality of metacestode material. Many different antigens (purified native, recombinant or synthetic) have been tested in the last decade, with debatable results suggesting that the heterogeneity of antigen preparation can negatively impact the sensitivity and specificity of the tests (Carmena *et al.*, 2006; Zhang *et al.*, 2012; Pagnozzi *et al.*, 2016). The inconsistency of tests results could be connected with the reduced inter-laboratory reproducibility of antigenic preparations which often rely on methodologies and different purification procedures. The use of different panels of sera can also contribute to the reduced reliability of results, i.e. due

Table 2. False positive reactions with sera of patients suffering from different parasitic/other infections and with control sera.

Parasitic/other infections	Antigens/ELISA tests used				
	<i>E. multilocularis</i>		<i>E. granulosus</i> s.l.		<i>Echinococcus</i> spp.
	AgEm	AgB	AgHF	<i>Echinococcus granulosus</i> (Bordier)	NovaLisa™ <i>Echinococcus</i> IgG (NovaTec)
Alveolar echinococcosis (n = 10)	n.c.	9	7	10	n.c.
Cystic echinococcosis (n = 17)	0	n.c.	n.c.	n.c.	n.c.
Ascariasis (n = 8)	0	0	0	1	0
Trichuriasis (n = 4)	0	0	0	0	0
Trichinellosis (n = 8)	0	0	0	0	1
Toxocariasis (n = 9)	0	0	0	1	0
Toxoplasmosis (n = 8)	0	1	1	0	0
Strongyloidiasis (n = 6)	0	0	2	1	0
Dirofilariasis (n = 1)	0	0	0	0	0
Rickettsiosis (n = 1)	0	0	0	0	0
Clinically healthy persons (n = 25)	0	2	1	2	2
Total false positive/tested	0/87	12/80	11/80	13/80	3/70

AgEm – somatic antigen of *E. multilocularis*; AgB – antigen B of *E. granulosus*; AgHF – hydatid fluid antigen of *E. granulosus*; n.c. - not calculated*

Table 3. Sensitivity, specificity and Youden index of tested antigens/tests.

Antigen/test	Parasitic disease	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)	YI
AgEm	<i>E. multilocularis</i>	90.0 (55.5–99.8)	100 (96.6–100)	100 n.c.	98.9 (93.1–99.8)	0.90
AgB		76.5 (50.1–93.2)	85.0 (75.4–91.4)	52.0 (37.7–66.0)	94.4 (87.8–97.6)	0.61
AgHF	<i>E. granulosus</i>	100 (83.8–100)	86.3 (76.7–92.9)	60.7 (47.2–72.8)	100 n.c.	0.86
<i>Echinococcus granulosus</i> (Bordier)		88.2 (63.6–98.5)	83.8 (73.8–91.1)	53.6 (40.5–66.2)	97.1 (90.1–99.2)	0.72
NovaLisa™ <i>Echinococcus</i> IgG (NovaTec)	<i>Echinococcus</i> spp.	77.8 (57.7–91.4)	95.7 (87.8–99.1)	87.5 (69.4–95.6)	91.8 (84.6–95.8)	0.74

AgEm – somatic antigen of *E. multilocularis*; AgB – antigen B of *E. granulosus*; AgHF – hydatid fluid antigen of *E. granulosus*;
CI – Confidence Interval; YI – Youden Index; PPV – Positive Predictive Value; NPV – Negative Predictive Value

to the lack of clinical characterization and appropriate classification of sera used for validation (Hernández-González *et al.*, 2012; Ito, 2013; Pagnozzi *et al.*, 2016).

ELISA based on *E. granulosus* s.l. hydatid fluid is reported to have a good or acceptable serological response in CE cases with liver and multiple organ locations (85 – 100 % sensitivities), but lung cysts gave very low sensitivity (50 – 60 %) (Gottstein & Reichen, 2002; Siracusano *et al.*, 2006). However, the antigen could not be considered as species-specific because its sensitivity for the detection of AE reached 90 – 97 % (Rafiei & Craig, 2002). Moreover, cross-reactions with many other helminthic infections were also observed (cestodes, nematodes and trematodes) (Eckert & Deplazes, 2004).

In our study, the antigens of sheep hydatid fluid of *E. granulosus* s.l. (AgHF) identified in all patients sera with surgically and molecularly confirmed *E. granulosus* liver cysts achieved 100 % sensitivity and 86.3 % specificity. The negative predictive values showed the highest rate (100 %). However, the positive predictive value was not optimal (60.7 %).

Antigen B, a partially purified lipoprotein initially described from *E. granulosus* hydatid cyst fluid (Oriol *et al.*, 1971), is commonly used in ELISA assays for AE and CE diagnostics (Schweiger *et al.*, 2012). Molecular and biological studies have revealed that a small subunit of AgB is also expressed in a metacestode of *E. multilocularis*, which may explain the high level of cross-reactivity with sera from the AE patients (Mamuti *et al.*, 2004). Therefore, serological tests utilizing AgB commonly shows a sensitivity of 63 – 92 % and a specificity of 85 – 93 %, with cross-reactions with antibodies in the sera of patients with AE infections (Eckert & Deplazes, 2004; Jiang *et al.*, 2012). AgB tested for the diagnosis of cystic echinococcosis by Reiterová *et al.* (2014) had 96.4 % sensitivity and 97.2 % specificity, with 93.1 % and 98.6 % for the positive and negative predictive value, respectively. Our findings confirmed that antigen B has the lowest sensitivity (76.5 %) and specificity

of 85.0 %, because it was identified in nine out of ten sera of AE positive patients. The positive predictive value showed a lower rate (52.0 %) than that described by Reiterová *et al.* (2014), while the negative predictive value had almost the same rate (94.4 %). A diagnostic sensitivity of 91 % and specificity of 82 % of the hydatid fluid antigen in the commercial ELISA *Echinococcus granulosus* test (Bordier Affinity Products, Crissier, Switzerland) was demonstrated by Poretti *et al.* (1999). In our study it showed to be 88.2 % sensitivity and 83.8 % specificity.

Given the results of all three *E. granulosus* antigens/tests analysed in the presented research and the results presented in other studies (Rafiei & Craig, 2002; Eckert & Deplazes, 2004; Jiang *et al.*, 2012; Reiterová *et al.*, 2014), the question regarding sensitivity and specificity for the diagnosis of alveolar echinococcosis could be opened. In general, sera positive to AgB and AgHF could be considered as *Echinococcus* spp. positive, and further serological, clinical and molecular or histopathological examinations should be performed do set the specific diagnosis.

The last tested diagnostic kit – NovaLisa™ *Echinococcus* IgG (NovaTec Immunodiagnostica, Germany) shows the detection of the IgG antibodies to *Echinococcus* spp. Despite the fact that this commercial test is commonly used in laboratories (Aydin *et al.*, 2018), studies on its sensitivity and specificity are very rare. The producer claims a high rate of diagnostic sensitivity (97.2 %) and specificity (99.4 %); however, in our study the test had a lower sensitivity (77.8 %) and specificity (95.7 %), and the PPV and NPV were 87.5 % and 91.8 %, respectively. In the study of Ahmad *et al.* (2017) the test showed a similar sensitivity value (75.0 %) when patients with liver hydatid cysts were tested and a lower value of 57.1 % in cases with hydatid cysts in the lungs.

In conclusion, the presented study showed the high diagnostic value of the somatic antigen to *E. multilocularis* (AgEm) antigen with a minimum of false positive or negative results. The tests for serological diagnostics of cystic echinococcosis were less sensi-

tive and specific, with more cross-reactions with sera of patients with AE or other parasitic diseases. The best results were obtained with antigens of sheep hydatid fluid. NovaLisa™ *Echinococcus* IgG (NovaTec Immunodiagnostica, Germany) proved to have similar values for diagnostic performance indicators as antigen B, except for the specificity, which was higher. Although a great effort is being made to develop highly sensitive and specific tests for the diagnosis of human echinococcosis, a combination of different serological methods and approaches in accordance with clinical and imaging findings is still essential to prove the correct diagnosis in suspected patients.

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Conflicts of Interest

The authors declare that no conflicts of interest exist.

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