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Evaluation of faecal flotation methods followed by species-specific PCR for detection of *Echinococcus multilocularis* in the definitive hosts

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Abstract

Echinococcus multilocularis is one of the most pathogenic zoonotic parasites in the temperate and arctic region of the Northern Hemisphere. For estimating the potential risk of human infection in endemic areas, reliable antemortem methods are needed to detect the parasite in carnivore definitive hosts. The sensitivity of routine flotation techniques for detection of *E. multilocularis* eggs was found to be low (3–33%) depending on the flotation solution used (specific gravities = 1.3–1.4). An improved faecal flotation followed by a species-specific PCR is described with a sensitivity of 74% (95% CI = 62–84%) and a specificity of 100% (95% CI = 94–100%). These parameters are similar to those of the intestinal scraping technique (sensitivity = 78%, specificity = 100%). The sensitivity of the improved flotation was significantly higher ($P < 0.0001$) than that of routine flotation techniques. The costs of the method are similar or lower than those of other antemortem diagnostic methods. Based on these data, the method is suitable for surveys of domesticated and wild carnivores.

Keywords

Echinococcus multilocularis, definitive host, carnivores, eggs, detection, flotation, PCR

Introduction

Human alveolar echinococcosis, caused by the metacestode stage of *Echinococcus multilocularis*, is one of the most pathogenic zoonoses in the temperate and arctic region of the Northern Hemisphere (Eckert *et al.* 2000). In the past 20 years, emergence, followed by spreading of the parasite, has been observed in Europe, and *E. multilocularis* is currently known to be endemic in 18 countries of the European Union (Casulli *et al.* 2010, Combes *et al.* 2012). Similar changes were also observed in North America in the past decades (Storandt and Kazacos 2012). Alveolar echinococcosis also represents one of the most significant zoonotic diseases in some parts of Asia (Vuitton *et al.* 2003, Torgerson 2013). The definitive hosts of this parasite are wild and domesticated carnivores. For estimating the potential risk of human infection in endemic areas, reliable methods are needed to detect *E. multilocularis* in definitive hosts. Surveys of wild carnivores (e.g., red foxes, raccoon dogs and coyotes) are often based on necropsies and direct examination of small intestines by sedimentation and counting technique or intestinal scraping technique. The sensitivity and specificity of these techniques are high (Deplazes and Eckert 2001). Surveys of domesticated and protected wild carnivores (e.g., dogs, wolves) cannot be based on postmortem examination (Martínek *et al.* 2001a,b). Copro-PCR methods can be used for the detection of *E. multilocularis* in these final hosts (Casulli *et al.* 2005, Jiang *et al.* 2012); however, the costs of these investigations are high (Mathis and Deplazes 2006). Faecal flotation methods might be alternative antemortem techniques; however, the sensitivities of routine flotation techniques for detection of *E. multilocularis* eggs are low. Moreover, eggs of *E. multilocularis* are morphologically indistinguishable from those of *Taenia* spp. and other *Echinococcus* spp. (Deplazes and Eckert 2001). Herein, we describe an improved faecal flotation followed by a species-specific PCR, which can be an alternative antemortem diagnostic approach based on sensitivity, specificity and costs.

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Materials and Methods

Collection of faecal samples

Carcasses of red foxes sent by hunters to the National Food Chain Safety Office, Budapest, from January 2002 to February 2013, in connection with the rabies immunization and control program, were included in this study (Sréter *et al.* 2003, 2004, 2005, Tolnai *et al.* 2013). Carcasses were forwarded in individual plastic bags at +4ºC. The intestinal tract was removed and stored at –20°C. For safety reasons, the intestinal tract was frozen at –80°C for 10 days before examination. After freezing, the gut was thawed at room temperature, the intestinal mucosa was collected and tested by sedimentation and counting technique (SCT) according to a previously published protocol (Deplazes and Eckert 1996). SCT can be considered close to a gold standard technique with 100% sensitivity and 100% specificity (Hofer *et al.* 2000). Adult worms of *E. multilocularis* were identified by morphology as previously described (Sréter *et al.* 2003). The intensity of infection was determined by counting of worms in low level infections (< 100 worms/fox) or by estimation of the worm number by counting in a known percentage of the sediment following dilution in high level infections (≥100 worms/fox). For molecular studies, 1–25 worms were collected from each infected foxes and stored in 70% (v/v) ethanol. The taxonomic status of the worms was also confirmed by multi-locus microsatellite analysis or a polymerase chain reaction (PCR) assay of the mitochondrial 12S rRNA gene (Casulli *et al.* 2010, Tolnai *et al.* 2013). Those animals from which PCR products were obtained were termed *E. multilocularis* infected. More than 10 g of faecal samples were taken from the colon of 73 foxes infected with *E. multilocularis*. More than 3 g of faecal samples were collected from the colon of further 65 foxes infected with *E. multilocularis*. Animals infected with both *Taenia* spp. and *E. multilocularis* were excluded from the study.

Faecal flotation

The sensitivity of the routine faecal flotations was tested with the faecal samples of 65 foxes (Tables I and II). Three grams of faeces from each fox were well mixed with either 16 ml of Breza solution (three parts of saturated $MgSO_4$ solution, three parts of saturated $Na_2S_2O_3$ solution and one part of water; specific gravity = 1.3) (n = 32) or modified Breza solution (one part of saturated $MgSO_4$ solution and one part of saturated $Na_2S_2O_3$ solution; specific gravity = 1.4) (n = 33) and strained through a sieve (0.6 mm mesh) by continuous and thorough mixing with a glass rod. The suspension was transferred to a 16-ml centrifuge tube and centrifuged at 2000 g for 3 minutes. Breza solution is one of the most slowly crystallizing flotation fluids and tends not to distort eggs. The eggs were removed from both tubes by touching the surface of the flotation fluid with the end of a glass rod and transferred to a microscopic slide. The drops on the slide were scanned under 100× magnification, and the number of eggs was recorded. When *Taenia*-type eggs were detected, the drops from the slides were washed to a 15-ml Falcon tube by water, and 1 ml of the upper part of the flotation solution from the centrifuge tubes was also transferred to the same Falcon tube. The tube was filled with 13 ml water and centrifuged at 2000 g for 10 minutes. The supernatant was discarded, the sediment was resuspended in 1 ml water, transferred to a 2-ml microtube and used for DNA purification and PCR.

Table I. Sensitivity of the routine faecal flotation using Breza solution with specific gravity of 1.3 followed by a species-specific PCR depending on the level of *Echinococcus multilocularis* infection

Level of infection (no. of worms per host)	No. of samples (no. of positive samples with flotation, no. of positive samples with PCR)	Sensitivity of the method (%) (95% CI)
Low (1–9)	16 (0, 0)	0 (0–21)
Medium (10–99)	10 (0, 0)	0 (0–31)
High (100–3,800)	6 (1, 1)	17 (3–64)
All (1–3,800)	32 (1, 1)	3 (1–16)

Table II. Sensitivity of the routine faecal flotation using modified Breza solution with specific gravity of 1.4 followed by a species-specific PCR depending on the level of *Echinococcus multilocularis* infection

Level of infection (no. of worms per host)	No. of samples (no. of positive samples with flotation, no. of positive samples with PCR)	Sensitivity of the method (%) (95% CI)
Low (1–9)	15 (3, 2)	13 (2–40)
Medium (10–99)	11 (4, 4)	36 (11–69)
High (100–5,500)	7 (5, 5)	71 (29–95)
All (1–5,500)	33 (12, 11)	33 (18–52)

Improved faecal flotation

The sensitivity of the improved faecal flotation was tested with the faecal samples of 73 foxes (Table III). Ten grams of faeces from each fox were well mixed with 32 ml of tap water and strained through a sieve (0.6 mm mesh) by continuous and thorough mixing with a glass rod. The suspension was transferred to two 16-ml centrifuge tubes and centrifuged at 2000 g for 10 minutes. The supernatant containing fat and dissolved pigments was discarded, and the sediment was resuspended by thorough mixing with a glass rod in 10 ml modified Breza solution (specific gravity = 1.4) in both tubes. The tubes were filled with modified Breza solution and centrifuged at 2000 g for 15 minutes. The eggs were removed from both tubes by touching the surface of the flotation fluid with the end of a glass rod and transferred to a microscopic slide. This step was repeated four times. The drops on the slide were scanned under 100× magnification, and the number of eggs was recorded. When *Taenia*-type eggs were detected, the drops from the slides were washed to a 15-ml Falcon tube by water, and 1–1 ml of the upper part of the flotation solution from the centrifuge tubes was also transferred to the same Falcon tube. The tube was filled with 13 ml water and centrifuged at 2000 g for 10 minutes. The supernatant was discarded, the sediment was resuspended in 1 ml water, transferred to a 2-ml microtube and used for DNA purification and PCR.

DNA purification and PCR

Samples stored in 2-ml microtubes were centrifuged at 2500 g for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 1.4 ml lysis buffer ASL of QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). After a disruption step performed in a TissueLyser LT bead mill (Qiagen) using 5-mm stainless steel beads for 5 min, DNA was extracted with QIAamp DNA Stool Mini Kit at an increased lysis temperature of 85°C according to manufacturer's instructions. Target DNA was amplified in a nested polymerase chain reaction (PCR) assay amplifying the mitochondrial 12S rRNA gene (Dinkel *et al.* 1998). PCR reactions were performed by using PuReTaq Ready-To-Go PCR beads (GE Healthcare, Buckingemshire, UK) with the conditions previously described (Dinkel *et al.* 1998). PCR products were separated by electrophoresis in 1.5% agarose gels and stained with ethidium bromide. Those faecal samples from which PCR products were obtained were termed *E. multilocularis* positive.

Statistical analysis

Diagnostic test evaluation, calculations of 95% confidence intervals (95% CI) and comparisons of the performance of diagnostic tests by Fisher's exact test were carried out by MedCalc 12.4 (MedCalc Software, Ostend, Belgium). The relationship between total worm counts and faecal egg count in fox faeces was analysed by calculation of the Spearman correlation coefficient using InStat 3.1 (GraphPad Inc., La Jolla, CA).

Results and Discussion

The use of faecal flotation followed by PCR is not a new idea for the detection of *E. multilocularis* infection in the definitive hosts (Reiterová *et al.* 2005, Kimura *et al.* 2013). However, evaluation of these methods has never been carried out, although the sensitivity of flotation techniques is known to be low for the detection of *Echinococcus* eggs. In our study, the sensitivities of routine flotation techniques for detection of *E. multilocularis* eggs were as low as 3% and 33% depending on the flotation solution used (specific gravities = 1.3 and 1.4) (Tables I and II). The sensitivity of routine flotation was significantly higher ($P < 0.05$), when modified Breza solution (specific gravity = 1.4) was used.

As the sensitivity of routine faecal flotation techniques is low for the detection of *E. multilocularis* eggs (Tables I and II), the protocol of the improved method was modified in five critical points. In routine flotation techniques, approximately 3 grams of faeces are used. In the protocol of the improved faecal flotation technique, the amount of faeces was increased to 10 grams. The faeces of carnivores generally contain a high amount of fat with dissolved pigments, which is responsible for frequent plug formation on the surface of the flotation solution. It can substantially decrease the sensitivity of faecal flotation. Therefore, an additional step of centrifugation with water was included before flotation. By this step, fat and dissolved pigments could be removed from the faeces, plug formation was rarely observed, and the sensitivity of the method could be improved further. Flotation solutions with specific

Table III. Sensitivity of the improved faecal flotation followed by a species-specific PCR depending on the level of *Echinococcus multilocularis* infection

Level of infection (no. of worms per host)	No. of samples (no. of positive samples with flotation, no. of positive samples with PCR)	Sensitivity of the method (%) (95% CI)
Low (1–9)	30 (18, 17)	57 (37–75)
Medium (10–99)	29 (25, 24)	83 (64–94)
High (100–22,000)	14 (13, 13)	93 (66–99)
All (1–22,000)	73 (56, 54)	74 (62–84)

Table IV. Characteristics of tests for postmortem and antemortem diagnosing *Echinococcus multilocularis* infection in definitive hosts (modified after Deplazes and Eckert 2001)

Diagnosis	Test	Sensitivity was compared to	Sensitivity (%) (95% CI)	Specificity (%) (95% CI) [b]	Sample no. that can be investigated per person and day	Costs of materials
Postmortem	Sedimentation and counting technique (SCT) (Hofer *et al.* 2000)	Gold standard method	100 (96–100) [a]	100 (96–100)	10	Low
	Sequential SCT (Umhang *et al.* 2011)	SCT	98 (94–100)	100 (98–100)	12	Low
	Intestinal scraping technique (IST) (Hofer *et al.* 2000)	SCT	78 (68–86)	100 (96–100)	20	Low
Antemortem	Coproantigen ELISA (Deplazes *et al.* 1999, Hartnack *et al.* 2013)	SCT; Copro-DNA PCR and AP	84 (71–92) 55 (41–69) [d]	99 (99–100) [c] 71 (65–77)	200	Medium
	Copro-DNA PCR (Dinkel *et al.* 1998, Hartnack *et al.* 2013)	IST; Coproantigen ELISA and AP	92 (87–95) 89 (79–96) [d]	100 (94–100) 93 (88–98)	15	High
	Sieving/PCR (Mathis *et al.* 1996, (Ziadinov *et al.* 2008)	SCT; AP	94 (80–99) 50 (29–72) [d]	100 (83–100) 100 (–)	15	Low-medium [e]
	Arecoline purgation (AP) (Ziadinov *et al.* 2008, Hartnack *et al.* 2013)	Copro-DNA PCR and Sieving/PCR; Copro-DNA PCR and Coproantigen ELISA	21 (11–34) 76 (55–94) [d]	100 (97–100) 100 (94–100)	10	Low
	Flotation/PCR (this study)	SCT	74 (62–84)	100 (94–100)	15	Low-medium [e]

[a]The sensitivity of 100% is only a theoretical assumption (Karamon *et al.* 2010), [b]As the intensity of infections was considerably different in these studies, sensitivities are not comparable. Moreover, as confidence intervals calculated on the basis of sample numbers were wide, the differences cannot be considered significant in the majority of cases, [c]On genus level, [d]Bayesian analysis in a latent class model was used to examine diagnostic performance. As necropsy was not included in these studies, low worm burdens might have been missed by all methods used. [e]Depending on prevalence of infection

gravity below 1.22 are not suitable for detection of *Echinococcus* and *Taenia* eggs as the gravity of the eggs of these species are above 1.22 (David and Lindquist 1982, Széll and Sréter unpublished). As a consequence of the high gravity of these eggs, high gravity flotation solutions and longer centrifugation times are needed to increase the sensitivity. Therefore, modified Breza solution (specific gravity 1.4) was used, and the centrifugation time was increased to 15 minutes. Finally, five drops of flotation fluid were transferred from both tubes to the microscopic slide, which also increases the chance of detection of eggs.

The above changes increased the sensitivity of improved faecal flotation for 74% (95% CI = 62–84%) (Table III). The sensitivity of the improved flotation technique was significantly higher ($P < 0.0001$) than that of routine flotation techniques (Tables I and II). No correlation was found between total worm counts and faecal egg count in fox faeces, which can be explained by the intermittent shedding of eggs (Kapel *et al.* 2006). This sensitivity is similar to, or lower than that of other postmortem and antemortem diagnostic techniques (Table IV). Even an identical diagnostic test may have a considerable difference in performance between different study populations (Hartnack *et al.* 2013). As the study populations were not identical, the comparison of the sensitivities of the various diagnostic techniques is difficult. In this study, the intensity of infection was lower (average worm count = 516) than that observed in the majority of studies (Mathis *et al.* 1996, Deplazes *et al.* 1999, Hofer *et al.* 2000). Although the confidence interval of sensitivity was generally not given in other studies, these figures were calculated and included in Table IV. Based on these calculations, the sensitivity of the method was almost identical with that of intestinal scraping technique (IST) (Table IV), which is frequently used in postmortem surveys (van der Giessen *et al.* 1999, Losson *et al.* 2003, Vervaeke *et al.* 2003, Duscher *et al.* 2006, Berke *et al.* 2008, Staubach *et al.* 2011). As the 95% CI of sensitivity of the majority of tests is wide, the differences of sensitivities are most likely not significant in most cases (Table IV). As none of the faecal samples collected from 60 uninfected foxes were positive by PCR (Dinkel *et al.* 1998), the specificity of the test can be considered 100% (95% CI = 94–100%) and is identical with that of the majority of antemortem and postmortem diagnostic tests. Based on sensitivity and specificity, the improved faecal flotation followed by a species-specific PCR can be an alternative antemortem diagnostic approach for surveys of domesticated and protected wild carnivores. The working cost of this method is similar to other antemortem and postmortem techniques (Table IV). The costs of materials are similar to or lower than other antemortem diagnostic techniques (Table IV). The improved flotation method can be carried out in all parasitology laboratories where routine coprological examination is done. Only the *Taenia*-type eggs should be collected and sent to a specialized molecular biology laboratory for species-specific PCR. When *Taenia*-type eggs are detected by the improved flotation technique, the drops from the slides should be washed to a 15-ml Falcon tube by water, and 1–1 ml of the upper part of the flotation solution from the centrifuge tubes should also transferred to the same Falcon tube. The tube should be filled with 13 ml water and centrifuged at 2000 g for 10 minutes. The supernatant should be discarded, the sediment should be resuspended in 1 ml absolute ethanol and transferred to a 2-ml microtube. These tubes can be transported to a molecular biology laboratory for PCR confirmation. This represents a clear advantage in developing countries, where molecular tools are not available in all laboratories and the infection rate of dogs is high (Vuitton *et al.* 2003, Torgerson 2013).

As no perfect gold standard is available for the antemortem diagnosing of *E. multilocularis* infection (Table IV), multiple tests should ideally be used in the population of interest (Hartnack *et al.* 2013). The improved faecal flotation might also be a candidate for multiple testing of domesticated and protected wild carnivore populations.

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