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Original article

Detection of *Echinococcus multilocularis* in faeces by nested PCR with the use of diluted DNA samples

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Abstract

The aim of this study was to choose the optimal variant of PCR examination of faeces to detect *Echinococcus multilocularis* infection which would allow to reduce the influence of different inhibitors in faeces. The investigation was carried out by comparison of 3 different methods of DNA isolation from faeces and different DNA dilutions used in PCR. Thirty five intestines of red foxes were used. Small intestines were examined by the sedimentation and counting technique (SCT). Faeces were collected from the rectum for PCR and flotation. DNA were isolated with the use of 3 different methods. Two methods were dedicated for faeces: method 1 (M1) – for larger samples and method 2 (M2) – for standard samples. The third method, method 3 (M3), was not dedicated for faeces. DNA samples were tested by nested PCR in 6 variants: not diluted (1/1) and 5 diluted (1/2.5, 1/5, 1/10, 1/20, 1/40). *E. multilocularis* was found by SCT in 18 from 35 (51.4%) intestines. *Taenia*-type eggs were detected only in 20.0% of faecal samples. In PCR the highest number of positive results (45.7%) were obtained during examination of DNA isolated by M1 method, and then 40.0% and 34.3%, respectively, for M2 and M3. In some samples positive results in PCR were obtained only in diluted DNA. For example, 8 from 12 positive samples isolated by M3 method gave the PCR negative results in non-diluted DNA and positive only after dilution 1:2.5, 1:10 or 1:20. Also 3 samples isolated by methods dedicated for stool gave positive results only after DNA dilution. The investigation has revealed that in copro-PCR for detection of *E. multilocularis* infection additional using of diluted DNA (besides non diluted) can avoid false negative results causing by PCR inhibition. In the best method of DNA isolation (M1), the use of non diluted DNA sample together with diluted in proportion 1:10 seems to be optimal.

Key words: *Echinococcus multilocularis*, PCR, copro-PCR, faeces

Introduction

Echinococcus multilocularis infection is one of the most dangerous zoonoses and still remains a significant public health problem. Definitive hosts of this tapeworms are carnivores (especially foxes, but also dogs and cats) which disperse the invasive eggs with faeces. A lot of prevalence study are conducted in populations of foxes and dogs, in order to estimate the infection risk for people. In wild carnivores post mortem examinations of small intestines are recommended – among them the sedimentation and counting technique (SCT) – regarded as the “gold standard”. But there are many situations when investigation must be carried out *in vivo* (for example examination of dog populations). Therefore, there are some techniques for detection of *E. multilocularis* infection in definitive hosts by faeces examination: coproantigen detection by ELISA or different PCR technique (Craig et al. 1995, Deplazes and Eckert 1996, Dinkel et al. 1998, van der Giessen 1999, Machnicka et al. 2003, Casulli et al. 2005, Reiterová et al. 2005, Dinkel et al. 2011, Mobedi et al. 2013). Among them PCR for copro-DNA detection is one of the most useful and sensitive. However, there are many problems connected with factors contained in faeces which can inhibit PCR reaction giving false negative results.

The aim of this study was to choose the optimal variant of PCR examination of faeces to detect *E. multilocularis* infection which would allow to reduce the influence of different inhibitors in faeces. Investigation was carried out by comparison of 3 different methods of DNA isolation from faeces and different DNA dilutions used in PCR.

Materials and Methods

Intestines (small and large) used in the investigation were obtained from 35 red foxes shot by hunters during official survey concerning the efficacy of anti-rabies vaccination. They were frozen at -80°C before examination to inactivate tapeworms eggs for at least 7 days.

Small intestines were examined by sedimentation and counting technique (SCT) (OIE 2008).

Samples of faeces were collected from the end of the rectum. Two gram samples from every intestines were frozen (-20°C) for using in molecular examination (PCR). In case of 25 intestines (which contained more faeces) additional faecal samples (2-3 g) were collected and examined for detection of *Taenia*-type eggs by McMaster technique in Raynaud modification (Raynaud 1970)

DNA isolation

DNA from samples were isolated and purified with the use of 3 different methods:

Method 1 (M1) – with the use of QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to producer protocol for larger volume of stool. In this protocol 1g of sample was firstly diluted (1:10) in lysing buffer and homogenized. Then, 2ml of mixture obtained were used in next stages of procedure of isolation and purification.

Method 2 (M2) – with the use of QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to standard producer protocol in which 200 mg sample was directly used in procedure of isolation and purification.

Method 3 (M3) – with the use of QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to standard producer protocol in which 20 mg sample was directly used in procedure of isolation and purification.

PCR

DNA isolated in each of 3 methods was tested by PCR in 6 variants: one not diluted (1/1) and 5 diluted (1/2.5, 1/5, 1/10, 1/20, 1/40). A nested PCR method was used, as described by Dinkel et al. (1998) with some modifications concerning reaction mixture and time conditions of amplification (Karamon et al. 2012). The sequence for amplification was a part of the *E. multilocularis* mitochondrial 12S rRNA gene. In a second stage of PCR the fragment specific for *E. multilocularis* (250 bp) was amplified.

The DNA from *E. multilocularis* adult worms derived from the intestine of foxes was used as the positive control. For specificity control the DNA was isolated from *Echinococcus granulosus* (protoscolices) and *Taenia hydatigena* (cysticercus tenuicollis). Distilled water was used as the negative control.

Results

E. multilocularis tapeworms were found in 18 from 35 (51.4%) intestines examined by SCT. The intensity ranged from 2 to 1055 worms per the intestine.

The PCR results (Table 1) taking account all DNA dilution variants were following: the highest number of positive results in PCR (16) were obtained during examination of the DNA isolated by the method 1 (M1) (variant for larger stool volume), and then 14 and 12, respectively, for (M2) and (M3) variants of isolation. In all samples negative in SCT there

Table 1. Selected results obtained by PCR for detection of *E. multilocularis* copro-DNA (in different DNA dilution and with the use of 3 different methods of DNA isolation) in comparison to the sedimentation and counting technique (SCT) and flotation.

Samples	SCT (No. of worms)	Flotation	PCR (M1)*						PCR (M2)*						PCR (M3)*						
			Dilutions of DNA used in PCR																		
			1/1	1/2.5	1/5	1/10	1/20	1/40	1/1	1/2.5	1/5	1/10	1/20	1/40	1/1	1/2.5	1/5	1/10	1/20	1/40	
1	+	(1055)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
2	+	(1500)	n	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	(750)	n	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	(650)	+	-	+	+	+	-	-	-	+	+	-	-	-	-	-	+	+	-	-
5	+	(150)	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+
6	+	(150)	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+
7	+	(126)	n	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	-	-	-
8	+	(125)	n	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	+	(45)	n	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-
10	+	(9)	n	+	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-
11	+	(8)	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-
12	+	(8)	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
13	+	(6)	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+
14	+	(5)	n	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	+	(4)	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+
16	+	(4)	n	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	+	(2)	n	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18	+	(2)	n	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+
19	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* Results of PCR obtained from DNA isolated by different methods: PCR (M1) – isolation by method 1 (M1) [QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany) – protocol for larger volume of stool]; PCR (M2) – isolation by method 2 (M2) [QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany) – standard protocol] PCR (M3) isolation by method 3 (M3) [QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) – standard protocol]. n- non-examined (lack of the sample).

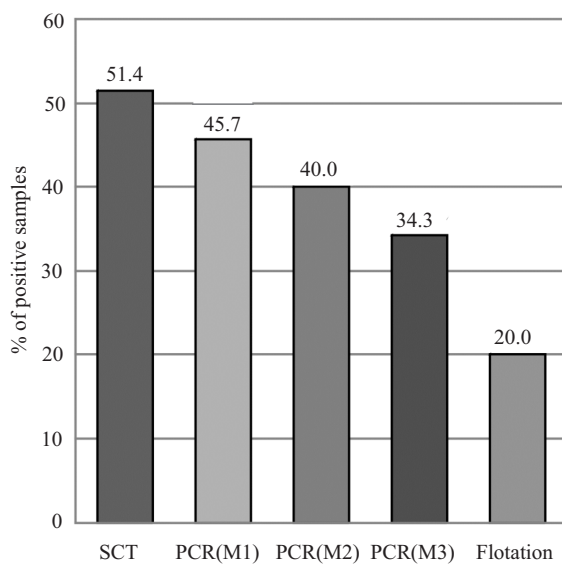


Fig. 1. The percentage of *E. multilocularis* positive samples detected by the sedimentation and counting technique (SCT), PCR (3 variants of DNA isolation) and *Taenia*-type eggs positive samples in flotation.

SCT – sedimentation and counting technique; PCR (M1) – isolation by method 1 (M1) [QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany) – protocol for larger volume of stool]; PCR (M2) – isolation by method 2 (M2) [QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany) – standard protocol]; PCR (M3) isolation by method 3 (M3) [QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) – standard protocol]; Flotation – detection of *Taenia*-type eggs by flotation.

were no positive PCR reactions in any variant, with only one exception: sample No. 19, negative in SCT, was estimated as positive in PCR (M1 isolation variant). In some samples, positive results in PCR were obtained only in diluted DNA. From 25 faeces examined by flotation only in 5 (20%) *Taenia*-type eggs were determined (Fig. 1).

Discussion

Blocking of PCR by inhibitors contained in faeces is the known problem in molecular diagnostics. When the concentration of inhibiting substances in the sample is high, the elimination of them during DNA purification (also by using dedicated methods) may be insufficient. In these cases significant amount of inhibitors leaves in the DNA sample and can block the PCR causing possibility of false negative results. Some authors demonstrated that samples which block the PCR may be identified by repeated examination of all negative ones with adding to each one additional positive control (Dinkel et al. 1998). But in such method all samples identified as inhibited had to be eliminated from further analysis at all, because there were still no answer which ones were really positive or negative.

The use of the DNA isolation kit containing the stage of inhibitors inactivation in our investigation

gave significantly better results than using the standard kit usually applied for isolation from tissues and blood. Among two variants of using the kit dedicated for stool a little better efficiency was observed in variant for larger volume of samples, probably because of preliminary homogenization of the sample.

Examination of different dilutions revealed significant impact of dilution of inhibitors contained in faeces on efficiency of the PCR. It was visible particularly distinctly in case of using the method 3 (M3) without the stage of inhibitors inactivation. Namely, 8 from 12 positive samples (67%) isolated by this method gave the PCR negative results in examination of non-diluted DNA. They were positive only after dilution 1:2.5, 1:10 or 1:20. However, it must be stressed that in case of 3 samples (No. 4, 6, 7), when methods of DNA isolation dedicated for stool were used (M1, M2), positive results were possible to obtain also only after DNA dilution. Lahmar et al. (2007) with the use of the same isolation kit (dedicated for stool) for detection of *E. granulosus* in dog faeces obtained very low efficacy, only 25% samples from infected dogs were positive in PCR. It could point out on the high concentration of inhibitors (DNA were not diluted) or lower effectiveness of PCR method used by the authors.

Therefore, dilution of the isolates in order to decrease concentration of inhibitors, decreases also the concentration of DNA. In case of samples characterized with high concentration of inhibitors but low concentration of specific DNA, dilution of isolates can lead to obtain false negative PCR results. Probably such cases were shown in 3 negative PCR results (despite using optimal DNA isolation method) which were obtained in samples of faeces collected from foxes infected with *E. multilocularis* (the presence of tapeworms in the intestines were confirmed by SCT).

Relatively popular method limiting the faecal inhibitors influence is the use for DNA isolation only eggs of tapeworms recovered from faeces by flotation. However, in our investigation *Taenia*-type eggs were found only in 5 samples from 9 positive in SCT and/or PCR. It showed that only about half of infected foxes would be examined by PCR and had a chance to be positive, when we used flotation as a method of inhibitors elimination. Another half would be eliminated from examination in the first step of procedure, flotation. So, such method of investigation could distinctly decrease real prevalence in the monitoring study. However, Al-Sabi et al. (2007) examining 15 experimentally *E. granulosus* infected dogs obtained higher efficiency of PCR with the use of DNA isolated from floated eggs

(Egg-DNA) than directly from faeces (Copro-DNA). Eighty percent of samples with low number of eggs per gram (EPG) from infected dogs were positive in PCR examination of Egg-DNA and only 47% in Copro-DNA. Poor results obtained with Copro-DNA probably resulted from the use of isolation kit not dedicated for faeces (without step of inhibitors binding) and examination of non-diluted DNA. It was confirmed by our investigation where during examination of not diluted DNA isolated by the method not dedicated for stool (M3) – only 1 from 5 samples (No. 1, 4, 5, 6, 13) in which *Taenia*-type eggs were detected was positive in PCR. Better results of flotation obtained by Al-Sabi et al. (2007) probably resulted from using the special combination of flotation followed by sequential sieving through nylon nets with mesh sizes of 31 and 20 μm . Therefore, it must be emphasized that other limitation of using the flotation as a first step of isolation is the examination of foxes infected with non-mature tapeworms which already have not produced eggs. In such cases copro-PCR is available to detect DNA contained in other parasitic elements, for example: tissue fragments or whole worms shed with the feces (Dinkel et al. 1998).

One sample (No.19), negative in SCT, was positive in PCR (DNA isolated by method 1 – M1). Despite SCT is regarded as “gold standard” in diagnosis of echinococcosis in final hosts, this method has also some diagnostic limitation (Karamon et al. 2010). Probably number of tapeworms in this intestines was below of limit of detection in SCT but concentration of specific DNA in examined portion of faeces was enough to obtain PCR positive result. It pointed out on the high sensitivity of this PCR procedure connected with proper method of DNA isolation. Moreover, the SCT method is also subject to the condition of the material used for examination (possibility of the autolysis of the parasites during prolonged exposure intestines at room temperature). Therefore, positive PCR result in SCT negative animals cannot be regarded as false positive (Casulli et al. 2005).

In conclusion, our investigation showed that in copro-PCR for detection of *E. multilocularis* infection additional using of diluted DNA (besides non diluted) could avoid false negative results causing by PCR inhibition. In the best method of DNA isolation (M1) the use of non diluted DNA sample together with diluted in proportion 1:10 seems to be optimal. But, when DNA isolation was done by the method not dedicated for faeces (M3), DNA samples required more and higher dilutions (because of higher inhibitors concentration).

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