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Research Note

Application of multiplex PCR for the simultaneous detection of *Taenia* spp.
from domestic dogs in the north of IranM. T. RAHIMI^{1,2}, S. SARVI^{1*}, A. DARYANI¹, M. SHARIF¹, E. AHMADPOUR³, A. SHOKRI¹, A. MIZANI¹

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Summary

The family Taeniidae is of great importance in the medical and veterinary fields, particularly in the tropics and subtropics. Identification of eggs of different *Taenia* spp. in the final host by morphological examination is difficult owing to their similarity. Therefore, a multiplex polymerase chain reaction (PCR) targeting a mitochondrial gene was applied to identify morphologically indistinguishable eggs. Fecal samples from 100 domestic dogs, from the Mazandaran province in Iran, were examined using the flotation/sieving method followed by multiplex PCR. Taeniid eggs were observed in 24 % samples, of which 12 %, 10 %, and 2 % were infected with *Echinococcus granulosus*, *Taenia* spp., and both *E. granulosus* and *Taenia* spp., respectively. *E. multilocularis* was absent in these samples. The prevalence of *E. granulosus* in the examined domestic dogs as definitive hosts in north of Iran was high (14 %). Therefore, people living in this region of Iran are in danger of acquiring hydatid cyst, which is a serious public health problem.

Keywords: Cestoda; *Taenia* spp.; *Echinococcus granulosus*; Multiplex Polymerase Chain Reaction (PCR)

Introduction

Taeniidae is the largest family of flatworms representing the order Cyclophyllidea. It includes many tapeworms with medical and veterinary importance (Qingling *et al.*, 2014). The adult stages of this family infect the intestine of humans (*Taenia*), and carnivores (*Taenia* and *Echinococcus*). Taeniidae parasites are distinguished by their life cycles that include a larval stage (metacestode) and a definitive host, which acquires the infection by ingestion of the metacestode containing protoscolices (Rakhshanpour *et al.*, 2012). The larval stages of some of these tapeworms can lead to severe disease or even death in the intermediate, accidental mammalian hosts, such as humans (Yang *et al.*, 2015). Two major species of tapeworms of medical, and public health importance are *Echinococcus granulosus* and *E. multilocularis*, which

cause cystic echinococcosis (CE) and alveolar echinococcosis (AE) in humans, respectively. Detection of *Echinococcus* infections in definitive hosts is of great importance to epidemiological surveillance and evaluation of echinococcosis control programs (Sarvi *et al.*, 2014). Control of echinococcosis relies on epidemiological information, including the accurate identification of the causative agents (Qingling *et al.*, 2014). Diagnosis of *Echinococcus* infections in canine hosts is problematic, as the eggs of taeniid cestodes are similar. Microscopic examinations of feces to identify these infections are therefore risky and non-specific (McManus, 2006). Polymerase chain reaction (PCR) is a highly sensitive approach to target mitochondrial DNA (mtDNA) sequences for both *Echinococcus*, and *Taenia* identification objectives, particularly in the discrimination of eggs (McManus, 2006). Among different PCR methods, multiplex PCR is considered useful for the detection of taeniid

eggs from both individual animals, as well as in epidemiological studies (Trachsel, *et al.*, 2007). A previous study (Yamasaki *et al.*, 2004) had successfully demonstrated a multiplex PCR assay for the differential diagnosis of taeniasis and cysticercosis in humans (*Taenia saginata*, *T. asiatica*, and *T. solium*). This technique is therefore very useful in the accurate identification of Taeniid cestode eggs, and overcomes the limitations of identification by morphology (Yamasaki *et al.*, 2004). Some studies have been carried out on wild and stray dogs; however, little attention has been paid to domestic dogs, which have been underestimated regarding their parasitic burdens and public health significance (Mehrabani *et al.*, 1999; Dalimi *et al.*, 2006). The objective of the current study was to apply multiplex PCR for differential diagnosis of *E. multilocularis*, *E. granulosus*, and *Taenia* spp. infections in domestic dogs in the north of Iran.



Fig. 1. Status of Mazandaran province in Iran

Materials and Methods

Study area

Mazandaran province (36° 33' 56" N 53° 03' 32" E) is placed at the northern part of Iran on the southern coast of the Caspian Sea (Fig. 1). It covers an area of approximately 23,842 km², and its population is 2,922,432. It has moderate and subtropical climate with a relative humidity of 70 % – 100 %, average temperature of 10 – 35 °C, and an annual rainfall of 800 – 1200 mm. The area is geographically classified into the coastal plains and the mountainous areas of the Alborz Mountain Range. The ecosystems of this province are diverse and comprise of many plains, prairies, and forests (Youssefi *et al.*, 2014).

Sample collection

Fecal specimens from 100 domestic dogs (male: 56; female: 44,

and ages <12 month: 21; ≥ 12 month: 79) were collected from the different areas of Mazandaran province, in the north of Iran, from January to September 2013. Each sample consisted of approximately 30 g of fresh stool collected from the rectum of domestic dogs, and was accompanied by information about the gender and age of the dog. The fecal samples were placed in labeled "ziploc" bags, and stored at -80 °C for at least seven days to reduce the risk of laboratory infection by inactivating any *Echinococcus* oncospheres and other infective materials. They were subsequently stored at -20 °C until used (Deplazes & Eckert, 1996).

Taeniid egg isolation with zinc chloride flotation and sieve method

To detect taeniid eggs, fecal samples from 100 domestic dogs were investigated by zinc chloride flotation and sieve method. Approximately 30 g of fresh stool was collected from the domestic dogs and 5 g of each sample was weighted and stirred into 50 ml distilled water until completely dispersed. The suspension was passed through four layers of gauze to remove large particles. The suspension was transferred into a 50 ml Falcon tube, and centrifuged at 1000 × g for 5 min. For isolating eggs, zinc chloride flotation (specific gravity: 1.45) was added to the sediment up to a final volume of 12 ml. Upon complete mixing, the mixture was centrifuged at 1000 × g for 30 min. The supernatant was passed through sequential sieves on 50-ml Falcon tubes with metal and polystyrene screens of mesh sizes of 37 and 20 μm, respectively (Deplazes & Eckert, 1996). The sieves were inverted and washed thoroughly with distilled water containing 0.2 % Tween 20. After adding phosphate-buffered saline (PBS; pH 7.2) to a final volume of 50 ml, the suspensions were centrifuged at 1000 × g for 30 min and the supernatant fraction was aspirated. The sediment of all samples was microscopically surveyed for the presence of taeniid eggs and positive samples were transferred into 1.5-ml tubes, and stored at -20 °C until used for further process.

DNA extraction

The DNA of taeniid eggs (samples with positive finding of eggs of *Taenia* spp.) was extracted using the QIAamp DNA Mini kit (Qiagen, Germany) according to the kit's protocol with slight modifications (Verweij *et al.*, 2001). DNA extraction was performed just on stool samples which were positive with zinc chloride flotation method. The sediment obtained from the above described egg isolation method was subjected to seven freeze/thaw cycles using liquid nitrogen and boiling water to disrupt the egg walls. Then, 200 μl of the sample was heated at 100 °C for 10 min. A volume of the ATL buffer containing 10 % proteinase K was added, completely mixed, and incubated for 2 h at 55 °C on a heat block. DNA extraction was continued according to manufacturer's instructions with the minor modification of increasing incubation time to 5 min at the final step to increase the yield of DNA. Finally, the concentration of the extracted DNA was measured by spectrophotometer, and DNA was stored at -20 °C until analysis.

Table 1. The applied primers characteristics for multiplex PCR

Parasite	Target	Name	Primer sequence (5'-3')	Amplicon size (bp)
<i>E. multilocularis</i>	nad1	Cest1	TGCTGATTTGTTAAAGTTAGTGATC	395 bp
		Cest2	CATAAATCAATGGAACAACAACAAG	
<i>E. granulosus</i>	rrnS	Cest4	GTTTTTGTGTGTTACATTAATAAGGGTG	117 bp
		Cest5	GCGGTGTGTACMTGAGCTAAAC	
<i>Taenia</i> spp.	rrnS	Cest3	YGAYTCTTTTTAGGGGAAGGTGTG	267 bp
		Cest5	GCGGTGTGTACMTGAGCTAAAC	

Multiplex PCR

Multiplex PCR was performed on the DNA from eggs as described previously (Trachsel, *et al.*, 2007). The multiplex reaction was designed to amplify a 395-base pair (bp) fragment of NADH dehydrogenase subunit 1 (nad1) of *E. multilocularis*, as well as 117 bp and 267 bp of a small subunit of ribosomal RNA (rrnS) of *E. granulosus* and other *Taenia* spp., respectively. Primers, conditions, and parameters for PCR were as previously described. Table 1 shows the used primers characteristics for Multiplex PCR in the current study. All samples were tested in 25 µl amplification reactions with 12.5 µl of the master mix (Qiagen Multiplex PCR, Germany), 2.5 µl of primers (2 µM of primers Cest1, Cest2, Cest3, and Cest4 and 16 µM of primer Cest5 in H₂O), 8 µl H₂O, and 2 µl template DNA. Additionally, for all PCR reactions, one negative control without DNA and one positive control with standard DNA (DNA extracted from adult worm of *E. granulosus*) were included to confirm the results of multiplex PCR. Finally, 10 µl of the PCR products were loaded on 1.5 % (W/V) agarose gels, and stained with safe stain (SYBR Green). PCR products were visualized by electrophoresis in TAE buffer (90 V for 30 min). The results of multiplex PCR were confirmed by single PCR using the primer pair Cest1/Cest2, Cest4/Cest5, and Cest3/Cest5 for *E. multilocularis*, *E. granulosus*, and *Taenia* spp., respectively (Stieger *et al.*, 2002; Trachsel *et al.*, 2007).

Statistical analysis

The data were analyzed using the SPSS 15 software. The chi-square test (χ^2 test) was used to determine associations between

the prevalence of Taeniid eggs and the dogs' age and gender. $P < 0.05$ was considered significant.

Results

Flotation technique

Taeniid eggs were observed in 24 % (N = 24) of the examined dogs' fecal samples in the north of Iran. Although male dogs revealed a higher infection rate of Taeniid eggs (24.9 %) compared to female subjects (22.7 %) and dogs aged less than 12 month showed a higher infection rate (27.4 %) than those aged more than 12 month (22.6 %), no statistically significant differences were observed in infection rates due to gender and age of examined individuals ($P > 0.05$).

Molecular analysis

The results of multiplex PCR on 24 DNA samples obtained from taeniid eggs for the amplification of a 117 bp fragment of rrnS indicated that 50 % (N=12) of the domestic dogs tested were infected with *E. granulosus*. Amplification of a 267-bp fragment identified *Taenia* spp. in 41.6 % (N=10) of the samples. Amplification of the 395-bp fragment of nad1 was not observed in any of the 24 fecal samples. In addition, two dogs were coinfecting with *E. granulosus*, and other *Taenia* spp. Table 2 shows information about the prevalence of infectivity of domestic dogs using multiplex PCR from DNA of taeniid eggs from their fecal samples in the north of Iran, according to sex and age.

Table 2. Prevalence of infectivity of domestic dogs in north of Iran using multiplex PCR from DNA of taeniid eggs from faecal samples according to sex and age (N=100)

Infection	Sex		Age		Total (n: 100)
	Male (n: 56)	Female (n: 44)	12 month > (n: 21)	12 month ≤ (n: 79)	
<i>E. granulosus</i>	8.9 % (5)	15.9 % (7)	14.2 % (3)	11.3 % (9)	12 % (12)
<i>Taenia</i> spp.	12.5 % (7)	6.8 % (3)	9.5 % (2)	10.1 % (8)	10 % (10)
<i>E. multilocularis</i>	0	0	0	0	0
Mixed infection (<i>E. granulosus</i> and <i>Taenia</i> spp.)	3.5 % (2)	0	4.7 % (1)	1.2 % (1)	2 % (2)
<i>p</i> value	>0.05		>0.05		
Total	24.9 % (14)	22.7 % (10)	27.4 % (6)	22.6 % (18)	24 % (24)

Discussion

In the current study, combining sieving and multiplex PCR, we show that 14 % of the examined samples were infected by *E. granulosus* in the Mazandaran province whereas the prevalence of *E. granulosus* in examined stray dogs from the north of Iran was reported 0 % (Gholami *et al.*, 2011). A similar work was carried out in the Chenaran region of Razavi Khorasan province in northeastern Iran. Fecal samples from 77 domestic and stray dogs, three foxes, ten jackals, and one wolf were examined. Infection with *E. granulosus* in the examined dogs was reported 26.3 % (Beiromvand *et al.*, 2011). In addition, in the Moghan Plains, 23.7 % (14) of domestic dogs were infected by *E. granulosus*, when assayed by nested PCR (Mobedi *et al.*, 2013).

Iran is considered an important endemic area of CE, and a wide range of intermediate hosts is commonly infected with *E. granulosus* (Sadjjadi, 2006). Annually, CE imposes a considerable burden to human society and animal husbandry. The total annual cost of CE in the country was estimated to be USD 232.3 million, including both direct and indirect expenses (Harandi *et al.*, 2012). The incidence of human CE is estimated to be 1.18 – 3 per 100,000 individuals in Iran (Rokni, 2008). The cost related to human CE was estimated to be USD 93.39 million. The cost of livestock CE was estimated to be USD 132 million (Harandi *et al.*, 2012), which includes economic losses due to the damage to livers and lungs found during inspections of carcasses, decrease in carcass weight, and reproductive losses.

E. multilocularis leads to a rare but lethal zoonotic disease (AE) in humans with limited options for successful treatment. Dogs as final hosts of *E. multilocularis* are important sources of this life-threatening infection for humans. Infected dogs are usually asymptomatic and can spread the eggs that can remain infective in the environment for months, even withstanding freezing (Veit *et al.*, 1995). Diagnosis is accomplished either by identifying the tiny adult worms after necropsy of dead dogs or by the molecular examination of stool of living dogs (Hildreth, *et al.*, 1991). In this study, the 395-bp amplicon of internal species-specific primers representative of *E. multilocularis* was not amplified in any of the examined samples. Our result is in agreement with a previously published study (Mobedi *et al.*, 2013). But another study detected *E. multilocularis* in carnivores in the Razavi Khorasan province in northeastern Iran (Beiromvand *et al.*, 2011). In our work, we found that 12 % of the dogs were infected with *Taenia* spp., while another group reported 21.8 % (16) of infection rate in the examined dogs (Beiromvand *et al.*, 2011).

An investigation in Lithuania showed significant differences between a modified McMaster method and the sieving-flotation technique (Bružinskaitė *et al.*, 2009). The sieving-flotation technique, which was applied in the present study, is the appropriate method for concentration and detection of Taeniid eggs in stool samples.

In the current study, optimized multiplex PCR was used as it is highly sensitive. Single taeniid eggs could be detected, which is in accord-

ance with other single-target, PCR-based tests (Abbasi *et al.*, 2003; Dinkel *et al.*, 1998). Multiplex PCR offers a great advantage for the discrimination of eggs in areas where *E. multilocularis*, *E. granulosus*, and *Taenia* spp. are all endemic, because it can detect mixed infections. The use of a highly sensitive method such as multiplex PCR is essential, particularly for *Echinococcus* species that tend to produce lower egg loads because of their lower biotic potential (Eckert, 2001). Furthermore, discrimination of *Echinococcus* strains and *Taenia* species from the isolated eggs can facilitate tracing the sources of infection and clarifying the life cycle of the parasites.

In conclusion, Iran is endemic for CE, and a high prevalence of *E. granulosus* is found in dogs. In addition, they can be considered as important source of parasitic infection for public health. Therefore, more studies are required to determine the prevalence of this parasite in domestic dogs in the north of Iran. Annually, the Mazandaran province attracts a large number of tourists owing to its popularity as a holiday destination, as well as immigrants and refugees for employment and financial activities. Thus, serious control and prevention of zoonotic diseases, particularly CE, is necessary in these areas.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of Interest

The authors declare that they have no competing interests.

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