

# Comparative assessment of rEPC1 antigen and copro-antigen for diagnosis of echinococcosis in dogs

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## Key words:

copro-antigen, diagnosis, dog echinococcosis, Dot-ELISA, recombinant EPC1 antigen

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## Abstract:

**BACKGROUND:** Diagnosis of *Echinococcus granulosus* in the definitive host particularly in dog is a significant complication in the endemic area. **OBJECTIVES:** The aim of this study is serological detection of *E. granulosus* in the infected dogs. **METHODS:** Dot-ELISA based on the copro-antigen and recombinant EPC1 antigen (rEPC1) for antibody detection was performed. Blood and fecal samples were collected from eleven treated puppies with 90000-100000 protoscolices (90% viability) and four treated puppies with distilled water as controls, on day before challenge and 7, 14, 21, 28 and 35 days post challenges. Furthermore, the blood and fecal samples were collected from 35 naturally infected dogs. **RESULTS:** In terms of experimentally infected dogs, sensitivity and specificity of Dot-ELISA were close for both antigens (copro-antigen, rEPC1) that were determined to be 100%, 88% for copro-antigen, and 100 and 94% for rEPC1, respectively. In the context of naturally infected dogs, our findings showed similar sensitivity in Dot-ELISA based on the anti-body detection (using rEPC1), and antigen detection (using copro-antigen), (100%), while these methods provided different specificity, about 75% for rEPC1 and 58% for copro-antigen. **CONCLUSIONS:** Our findings indicated that both antigens are qualified. REPC1 antigen is not able to detect the infection during the first 15 days post-infection, whereas the antibody cannot be detectable. REPC1 protein may work for screening of *E. granulosus*, while copro-antigen can be useful for diagnosis of current acute infection. However, both methods are recommended for screening of sheepdog, guard dogs and police dogs.

## Introduction

*Echinococcus granulosus* is considered as one of the most significant parasitic infections throughout the world as a causative agent of cystic hydatid disease which is transmitted between canines and numerous herbivorous livestock animals as inter-

mediate hosts (das Neves et al. 2017). It is thought to be an important global parasitic disease of humans and animals. Cystic echinococcosis (CE) is endemic in Iran, where a variety of animals act as intermediate hosts (Eslami and Hosseini 1998; Umhang et al. 2013). Fasihi Harandi et al, (2012) estimated annual surgical incidence of CE (Cystic

echinococcosis) with a rate of 1.27/100,000 population from 2000-2009 in Iran. Furthermore, average annual cost of CE in Iran was estimated at US\$232.3 million, including both direct and indirect costs (Fasihi Harandi et al. 2012). The cost associated with human CE was estimated at US\$93.39 million and the annual cost related to CE in livestock was estimated at US\$132 million (Fasihi Harandi et al. 2012), indicating the importance of infection control. Therefore, detection of *E. granulosus* in the definitive host is an important problem in endemic areas. Control of infection in dogs is much cheaper than those in the intermediate host. The diagnosis of hydatid cyst is mainly focused on human but diagnosis and screening of infection in dogs is most important in endemic area for control programs, and also can be useful for assessing the dynamics of hydatidosis transmission (Carmena et al 2006, Allan and Craig 2006). The diagnosis of canine echinococcosis can be a challenge in surveillance of control programs, because there is no perfect gold standard test. Several diagnostic methods have been employed for diagnosis of *E. granulosus*, including necropsy of dogs, examination of the small intestine, coprological examinations and purging of dogs with arecoline hydrobromide (Ibrahim, 2017). Routine stool exam cannot differentiate the eggs of echinococcus from other taenia species due to morphological similarity (Dinkel et al, 1998). These techniques are time consuming, labor intensive, hazardous and suffer from low sensitivity (Jenkins et al. 1990). The immunological methods such as ELISA and dot-blotting are highly used, although their accuracy is largely dependent on the specificity and sensitivity of antigens (Carmena et al 2006; Wachira et al

1990). Despite the development of sensitive and specific methods, the immunodiagnosis of CE and echinococcosis remains a complex task (Ortona et al 2003; Siracusano and Bruschi 2006). Majority of the available screening tests can produce a high percentage of false-negative results (up to 25 %), as well as false-positive results which occur using different assays and can be caused by co-infection with other cestodes or helminths (Carmena et al 2006). Recombinant antigens and synthetic peptides can be useful applications for human hydatidosis as specific peptides (Hernandez-Gonzalez et al 2008). Recombinant EPC1, a 8.5-kDa antigen from *E. granulosus*, has been shown to be effective for diagnosis of human hydatidosis (Li et al 2003; Cai et al 2011), without any report about the usefulness of this recombinant antigen for detection of dog echinococcosis. Dogs have an immune response against the adult parasites (Zhang et al 2003), indicating that serological tests using specific antigens may be useful. The current study was aimed to assess the efficacy of copro-antigen detection and antibody detection (based on the rEpC1) in the diagnosis and monitoring of echinococcosis.

## **Materials and Methods**

**Positive and negative reference serum samples:** We performed a cross-sectional study on 15 dogs (2-3 months old). Dogs were raised from birth at the Small Animal Hospital, Faculty of Veterinary Medicine, University of Tehran. They were maintained on commercial dog food and water. Puppies were vaccinated against distemper, Rabies virus, parvoviruses and leptospirosis and treated orally with Praziquantel. Puppies

were kept in separate cages with complete sanitary conditions and were divided into two groups. The first group (11 puppies) was inoculated with 90000-100000 (viability more than 90%) protoscoleces. Protoscoleces were collected from fertile hydatid cysts of ovine liver. Each dog was fed about 100000 protoscoleces. In the second group, 4 puppies were selected as a negative control group, and were kept in the same condition, but not fed any protoscolex. It is worth noting that worm infections were not found in puppies of the control group. In addition, no change in blood parameters and clinical signs were seen in experimentally infected puppies and control group.

Stool sampling was performed six times including the day before challenge, 7, 14, 21, 28 and 35 days post infection. In addition, blood was collected from dogs in these days. The blood was clotted at room temperature for 30 min and then at 4 oc for 4 h. The clot was separated from the serum by centrifugation at  $3,000\times g$  for 10 min and the serum stored at -20 oc, until use.

**Other parasites infection (Naturally infected dogs):** The small intestines of 35 stray dogs were opened and immersed in warm PBS. Detached worms and the intestinal contents were passed through sieves, and worms were enumerated under a bright light on a black background (WHO, 2006). The blood and fecal samples were collected from dogs with natural infection. Inspection of small intestine of these dogs showed that dogs were infected with other carnivores' intestinal worms.

**Echinococcus granulosus copro-antigen:** strips were dipped into 1: 50, 1; 100, 1; 250, 1; 500, 1; 1000 dilution of dogs sera (5 00  $\mu$ l) and placed on shaker for 1 h. The strips were then washed 3 times in PBS-T

for 5 min. Then, 100  $\mu$ l of horseradish peroxidase conjugated rabbit anti-dog IgG (Sigma\_Aldrich) at a 1:10000 dilution was added at 1: 2500 dilution and placed for 1 h on shaker in dark place. After rinsing, peroxidase reaction was visualized with 0.06% (w/v) diaminobenzidine tetrahydrochloride in 50 mM Tris-HCl (pH 7.6) and 0.03% (v/v) H<sub>2</sub>O<sub>2</sub>. The reaction was stopped after 2 min with distilled water.

The sensitivity, specificity and efficacy were calculated as follows:

Sensitivity=Number of true positives/  
Number of true positives+Number of false negatives

Specificity=Number of true negatives/  
Number of true negatives+number of false positives

Efficacy=Sensitivity+specificity/2

## Results

**Copro-antigen Dot-Elisa in experimentally infected dog:** In this method, copro-antigen showed positive reaction on days 15, 28, 35 after challenge and also control dogs showed a negative reaction.

Sensitivity and specificity of Dot -ELISA based on the copro-antigen for diagnosis of experimentally infected dogs were 100% and 88%; moreover, the efficacy of copro-antigen was 95.5% (Table 1 and Fig. 4).

**Copro-antigen Dot-Elisa in naturally infected dogs:** In naturally infected dogs with other carnivores' intestinal worms, copro-antigen showed sensitivity and specificity of 100% and 58% (the gold standard was necropsy of dogs). Copro antigen-based tests have a greater number of false positives which reduces the specificity of the test. Moreover, efficacy of copro- antigen was 70.5% (Table 1 and Fig. 5).

Table 1. Comparison of sensitivity, specificity, efficacy, positive and negative predictive values of Copro-Ag dot-ELISA and rEPC1 Ag dot-ELISA in detection of dog echinococcosis.

Type of Ag	Experimentally infected dogs with <i>Echinococcus granulosus</i> No.11	Healthy Puppies No.4	Sera of puppies before challenge (negative control sera) No.11	Sera of dogs with other parasites (Natural infection) No.35	True positive	False positive	True negative	False negative
Copro-Ag dot-ELISA	11 (positive sera)	0 (positive)	2 (positive sera)	34 (positive sera)	11	36	50	0
rEPC1 Ag dot-ELISA	11 (positive sera)	0 (positive)	1 (positive sera)	16 (positive sera)	11	17	50	0

**REPC1 Dot-Elisa in experimentally infected dogs:** Dogs with a serum dilution of 1: 50, 1; 100, 1; 250, 1; 500 and 1: 1000 were studied by Dot-ELISA. Maximum and minimum color spots were observed at a dilution of 1:50 and 1:1000. Sera of infected dogs showed positive reaction at all dilutions and also against the rEPC1. Furthermore, control group showed a negative reaction.

Furthermore, sensitivity and specificity of Dot -ELISA based on the rEpC1 for serodiagnosis of experimentally infected dogs were 100% and 94%. Moreover, the efficacy of rEpC1 Ag was calculated to be 95.5% (Table 1 and Fig. 4).

**REPC1 Dot-Elisa- in naturally infected dogs:** REPC1 showed a sensitivity and specificity of 100% and 75%, with naturally infected sera (other carnivores intestinal worms). The gold standard was necropsy. Furthermore, efficacy of rEPC1 was 87.5 (Table 1 and Fig. 5).

## Discussion

The diagnosis of hydatid cyst is mainly focused on human, but the diagnosis of infection in dogs (adult worm) is most important in endemic areas for surveillance of control programs, also it can be useful for assessing the dynamics of hydatidosis

transmission (Dinkel et al. 2011, Allan and Craig 2006). Two main immunodiagnostic approaches for diagnosis of *E. granulosus* infection in definitive hosts are antibody detection and copro-antigens detection in feces. The most common diagnostic tests in dog are based on copro-antigen detection technique. Copro-antigen ELISA by using antibodies against *Echinococcus proglottid* somatic antigens and or excretory/secretory (ES) antigens are the most practical approach for the diagnosis of the intestinal *E. granulosus* infection in dogs (Mastin et al, 2015; Deplazes et al, 1992; Jenkins et al, 2000; Dinkel et al., 2011). Moreover, copro-antigen ELISA is far more sensitive than arecoline and can be used for *E. granulosus* detection in dog populations in both laboratory and field conditions (Jenkins et al, 2000; Eckert and Deplazes, 2004). The sensitivity and specificity of copro-antigen ELISA for detection of *E. granulosus* infection in canids permits the detection of the parasite during the prepatent period (Ahmad and Nizami, 1998). As matter of fact, it can show the current status of the infection (Jenkins et al., 2000), whereas its results correlated with the worm burden in the dog intestine (Mastin et al, 2015). In the present study, none of the experimentally infected dogs had clinical sign and changes in blood parameters also were shown to be negative

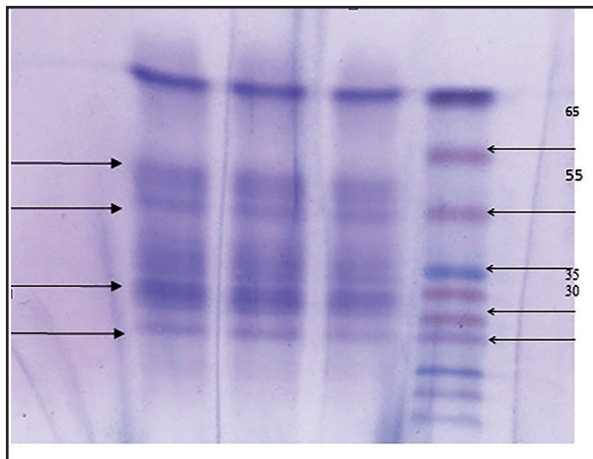


Figure 1. SDS-PAGE of Copro-antigen with 12% concentration under denatured conditions. Four bands with molecular weights of 30, 35, 55 and 60 KDa were observed.

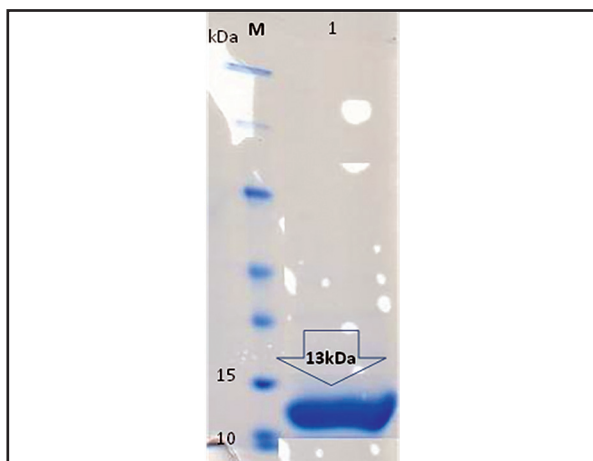


Figure 2. SDS-PAGE of rEPC1 antigen with 12% concentration under denatured conditions. A band with 13KDa molecular weight was observed.

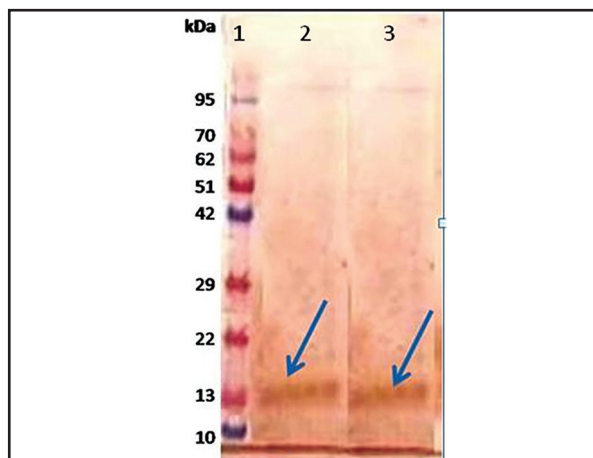


Figure 3. Reactions of rEPC1 antigen (13kDa subunit) with *E. granulosus* positive serum samples (1:50 and 1:100, Western blotting).

after a lag phase of up to 2 weeks. This initial delay in copro-antigen production may be related to the development of the worms in intestine (Al-Jawabreh, 2015; Jenkins et al, 2000). The researchers have suggested that the amount of copro-antigen and its diagnosis is heavily dependent on burden of infection, indicating the disadvantage of method based upon copro-antigen (Deplazes et al, 1992; Jenkins, et al, 2000; Allan and Craig, 2006; Allan and Craig, 1989; Allan et al, 1992; Jenkins et al, 2000). In this study, sera from puppies infected in the laboratory were used to determine the diagnostic sensitivity of copro-antigen and simultaneously, the sera of naturally infected dogs were used to determine the diagnostic specificity of copro- antigen. The sensitivity of copro-antigen detection in this study was acceptable. Its sensitivity has been reported to be generally good with moderate to high worm burdens (>100 worms), but less in animal with low worm burdens (Pierangeli et al., 2010). The specificity of Dot -ELISA based on the copro-antigen for diagnosis of experimentally infected dogs, naturally infected dogs with other carnivores' gastrointestinal worm parasites were 88%, and 58%. Copro antigen is highly specific and can be detected by antibody in experimentally infected dogs by days 5-10 post infection, and therefore does not depend on the presence of eggs (Deplazes et al, 1992). Allan et al, (1992) reported 96% specificity for copro-antigen and good sensitivity (77-88%) based on confirmation by arecoline purge (Mastin et al, 2015; Lopera et al, 2003). The sensitivity and specificity of copro-antigen Dot-ELISA was good in experimentally infected dogs by high burden of infection (Craig, 1995; WHO, 2006). However, the specificity was relatively low in naturally infected dogs

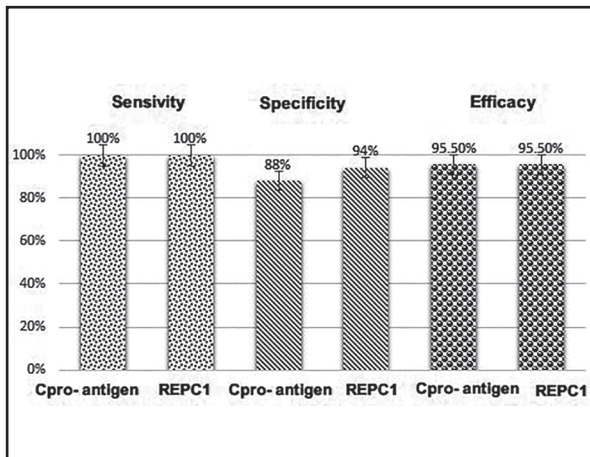


Figure 4. Comparison of sensitivity, specificity and efficacy of copro-Ag Dot-ELISA and rEPC1 Dot-ELISA for detection *E. granulosus* infection in experimentally infected dogs.

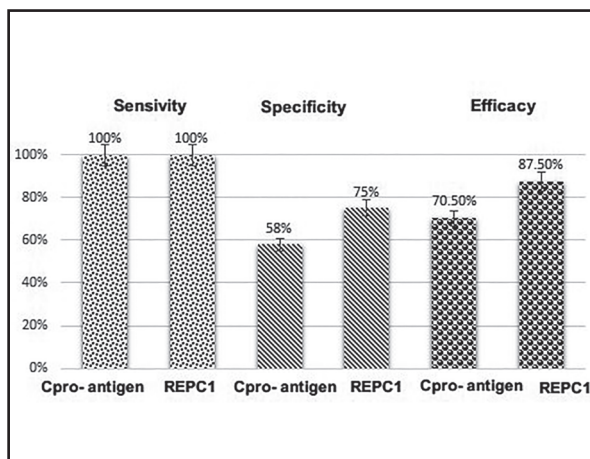


Figure 5. Comparison of sensitivity, specificity and efficacy of Copro-Ag Dot-ELISA and rEPC1 Dot-ELISA for detection of *E. granulosus* infection in naturally infected dogs.

which indicates the actual performance in naturally infected dogs is less certain than experimentally infected dogs, because of potential cross reactivity with antigens from *Taenia* species or other helminths and needs to be defined in fields. It should be noted that sensitivity and specificity have often been obtained in experimentally infected dogs. However, test parameters will vary with the population. We hope the method in this study will be optimized in the future with a comprehensive study which is aimed at screening of dogs. As evident from the recent literature, recombinant antigens and

fusion peptides can be useful for diagnostic applications mainly in humans as specific peptides (Zhang et al. 2003). In this study, recombinant protein (*Echinococcus protoscolex* gene) EPC1 was used as antigen for the detection of specific antibodies of *E. granulosus* in dogs. Therefore, the ability of recombinant protein EPC1 in dog serum samples was also analyzed by Dot-ELISA. Specific serum antibodies were shown to be detectable in the serum of dogs after experimental infection with *E. granulosus* using metacestode antigen and others confirmed the appearance of specific antibodies by using antigens derived from the oncosphere in ELISA (Barriga, 1986, Singh and Dhar, 1988; Sixl et al, 1988). Our results showed that sensitivity and specificity of Dot-ELISA based on the rEPC1 Ag for serodiagnosis of experimentally infected dogs, naturally infected dogs were 100%, 94%, 100% and 75%, respectively. This result indicated a high sensitivity compared with previous studies for detection of serum antibodies that showed variable sensitivities, ranging from 40 to 90% (Benito et al, 2001; Gasser et al, 1994; Mastin et al, 2015; Jenkins et al, 1990), and also cross-reactivity with other parasite species may occur (Gasser et al, 1988), while sensitivity was reported to be high (73%) for natural canine *E. granulosus* infection using a protoscolex antigen-ELISA in south-east Australia, there was no correlation with worm burden. Gasser et al (1990) reported that a recombinant *E. granulosus* protoscolex antigen was 100% specific for *E. granulosus* antibodies in dog sera, but sensitivity was significantly low for the native protoscolex antigen that is in contrast with our study. But the specificity was relatively low in naturally infected dogs, suggesting the actual perfor-

mance of antigen in naturally infected dogs is less certain than experimentally infected dogs. In our study, the relative good efficacy of rEPC1 Ag enables useful application in determination of presence or absence of *Echinococcus* spp. and estimating relative exposure rates in dog populations. However, antibodies persist after the elimination of the worm burden, accordingly, antibody prevalence does not correlate with the actual prevalence. Furthermore, antibody detection is not correlated with the worm burden, while copro- antigen detection is indicative of acute infection (Pierrangeli et al, 2010). However, rEPC1 antigen seems to work for screening of infected dogs. On the other hand, copro-antigen production is easy and inexpensive but the major limitations of copro-antigen are associated with the *E. granulosus* worm burden. Nonetheless, copro-antigen efficiency is important for the detection of current infection of dogs with *E. granulosus*. The specificity of copro-antigen was relatively low in naturally infected dogs than experimentally infected dogs, indicating the actual performance in naturally infected dogs can be decreased, which can also be true for rEPC1 antigen. We proposed that if the goal is screening of *E. granulosus*, rEPC1 may work for this purpose, but if the goal is detection of current infection, copro-antigen can be applicable in this matter.

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## ارزیابی مقایسه‌ای آنتی ژن rEPC1 و آنتی ژن‌های مدفوعی در تشخیص اکینو کوزیس در سگ‌ها

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### چکیده

**زمینه مطالعه:** تشخیص اکینو کوزیس در سگ بعنوان میزبان نهایی یکی از مهمترین چالش‌های این آلودگی بویژه در مناطق اندمیک آن است. **هدف:** هدف از مطالعه حاضر تشخیص اکینو کوزیس در سگ‌های آلوده است. **روش کار:** بدین منظور روش الایزای نقطه ای (DOT-ELISA) برای شناسایی آنتی ژن‌های مدفوعی، همچنین با استفاده از آنتی ژن نوترکیب rEPC1 برای شناسایی آنتی بادی‌های ضد انگل مورد استفاده قرار گرفت. در مطالعه حاضر ۱۱ توله سگ با ۱۰۰۰۰۰-۹۰۰۰۰ پروتواسکولکس (۹۰٪ زنده) و ۴ توله سگ با آب مقطر چالش شدند. نمونه‌های خون و مدفوع توله‌ها قبل از چالش و روزهای ۳۵، ۲۸، ۲۱، ۱۴ و ۷ پس از چالش جمع‌آوری شد. نمونه‌های خون و مدفوع از ۳۵ سگ که بطور طبیعی سایر آلودگی‌های کرمی داشتند نیز جمع‌آوری گردید. **نتایج:** در سگ‌هایی که بطور تجربی در آزمایشگاه آلوده شده بودند، حساسیت و ویژگی آزمون الایزای نقطه‌ای با هر دو آنتی ژن بهم نزدیک بود و بترتیب ۱۰۰٪ و ۸۸٪ برای کوپرو آنتی ژن و ۱۰۰٪ و ۹۴٪ برای rEPC1 بدست آمد. در سگ‌هایی که بطور طبیعی آلودگی به سایر کرم‌های انگلی داشتند با استفاده از آنتی ژن نوترکیب rEPC1، حساسیت ۱۰۰٪ و ویژگی ۷۵٪ بدست آمد. در این گروه سگ‌ها آنتی ژن‌های مدفوعی حساسیت ۱۰۰٪ و ویژگی ۵۸٪ را نشان دادند. **نتیجه‌گیری نهایی:** این نتایج نشان می‌دهد که هر دو آنتی ژن در تشخیص آلودگی کارایی قابل قبول دارند. آنتی ژن نوترکیب قادر به تشخیص آلودگی طی ۱۵ روز اول پس از آلودگی نیست، زیرا هنوز آنتی بادی به میزان قابل اندازه‌گیری نرسیده است، این آنتی ژن می‌تواند برای آزمون‌های غربالگری مورد استفاده قرار گیرد. بمنظور تشخیص آلودگی بویژه در سگ‌های گله، سگ‌های نگهبان و سگ‌های پلیس که ارزشمند هستند، بکارگیری توأم هر دو آنتی ژن توصیه می‌شود.

**واژه‌های کلیدی:** آنتی ژن مدفوع، تشخیص، اکینو کوزیس در سگ، الایزای نقطه‌ای، آنتی ژن نوترکیب EPC1

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