



Veterinary Microbiology

Sensitivity, specificity and comparison of three commercially available immunological tests in the diagnosis of *Cryptosporidium* species in animals



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ABSTRACT

The study was conducted to compare the specificity of immunological diagnostic methods used for the diagnosis of *Cryptosporidium* species capable of causing life-threatening infection in both immunosuppressed and immunocompetent patients. For the detection of *Cryptosporidium* species in 79 animals with diarrhoea, we used three Copro-antigen tests: RIDASCREEN[®] *Cryptosporidium* test, *Cryptosporidium* 2nd Generation (ELISA) and RIDA[®] QUICK *Cryptosporidium*. For immunoassays we used positive and negative samples detected by means of polymerase chain reaction and validated by sequencing and nested polymerase chain reaction to confirm the presence six different species of *Cryptosporidium* species. Prevalence of cryptosporidiosis in the entire group determined by enzyme immunoassay, enzyme linked immunosorbent assay, immuno-chromatographic test and polymerase chain reaction was 34.17%, 27.84%, 6.33% and 27.84%, respectively. Sensitivity of animal samples with enzyme immunoassay, enzyme linked immunosorbent assay, and immuno-chromatographic test was 63.6%, 40.9% and 22.7%, resp., when questionable samples were considered positive, whereas specificity of enzyme immunoassay, enzyme linked immunosorbent assay and immuno-chromatographic test was 75.9%, 78.9% and 100%, respectively. Positive predictive values and negative predictive values were different for all the tests. These differences results are controversial and therefore reliability and reproducibility of immunoassays as the only diagnostic method is questionable. The use of various *Cryptosporidium* species in diagnosis based on immunological testing and different results obtained by individual tests indicate potential differences in Copro-antigens produced by individual *Cryptosporidium* species.

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Introduction

Cryptosporidia are cosmopolitan widespread parasite, with broad host specificity, primarily occurring in young livestock. Humans are also susceptible, especially immunodeficient individuals. In the recent years, considerable attention has been paid to cryptosporidiosis caused by zoonotic species, especially to host specificity of these species, and the associated possibility of disease transmission between different hosts in the environment.

Clinical manifestation of cryptosporidiosis comprises asymptomatic forms but also severe chronic states causing damage to the gastrointestinal tract and accompanied by diarrhoea, anorexia, cachexia, dehydration with dissemination of parasites to the surrounding organs with potential fatal impact on immunosuppressed subjects.^{1,2} Cryptosporidiosis with clinical manifestation as well as asymptomatic shedding of oocysts is more frequent in the young than in adults. Reliable and early diagnosis is required not only of infection with fatal consequences but also of asymptomatic infections.^{3,4}

A direct microscopic diagnosis of *Cryptosporidium* from stool samples is laborious and requires qualified personnel to identify the pathogen. The diagnostic accuracy is significantly reduced by a low concentration of oocysts or by mechanically/enzymatically damaged oocysts and irregularly excreted oocysts.^{5,6} Worldwide seroprevalence in livestock reaches 27–30%.⁷

In the recent years, Copro-antigen commercial tests, such as enzyme immunoassay (EIA), or immunochromatographic dipstick test (ICT) have been used for rapid diagnosis. According to the manufacturer, these tests are rapid and sensitive enough, but provide only quantitative results which suffice only for detection of the presence of pathogen in the holdings but not for individual diagnosis and identification.^{6,8} Diagnosis based on Copro-antigen as a single test for detecting the presence of cryptosporidia is inadequate, particularly in risk groups such as immunodeficient patients with life-threatening diarrhoea.⁹ Therefore, molecular methods, including polymerase chain reaction (PCR), became reference methods for the detection, identification, differentiation and generic genotyping of *Cryptosporidium* spp.^{10,11}

The aim of our study was to evaluate and compare three commercially available Copro-antigen tests, namely RIDASCREEN[®] *Cryptosporidium* test (Enzyme Immunoassay – EIA), *Cryptosporidium* 2nd Generation (Enzyme Linked Immunosorbent Assay – ELISA), and RIDA[®] QUICK *Cryptosporidium* (Immuno-chromatographic test – ICT). PCR method, sequencing and phylogenetic analysis were used to confirm the presence of cryptosporidia and disprove false positivity and negativity of samples.

Materials and methods

Study population – samples

Stool samples were collected from 79 animals divided into three groups (1st – 35 pigs; 2nd – 34 calves; 3rd – 10 lambs),

with clinical symptoms (diarrhoea, abdominal pain, anorexia, weight loss, dehydration).

By means of PCR analysis and subsequent sequencing, we truly detected positive and negative samples that were used for the immunoassay. We used 22 positive samples of varying localization. Intestinal species: calves – *C. parvum* (10); *C. bovis* (2); pigs – *C. scrofarum* (5), *C. suis* (2); and gastric species: pigs – *C. muris* (2), *C. andersoni* (1). The sensitivities, specificities, positive predictive values and negative predictive values were calculated according to Loong.¹²

Molecular analysis

DNA isolation

Genomic DNA was extracted from 100 mg of stool sample using a DNA-Sorb-B Nucleic acid Extraction kit (AmpliSence, Russia) according to the manufacturer's instructions. Before extraction, we homogenized the stool and disrupted oocysts at 6500 rpm for 90 s with addition of 0.5-mm-glass beads, 1.0-mm-zircon beads and 300 μ L lysis solution in a homogenizer Precellys 24 (Bertin technologies). Purified DNA was stored at -20°C until use in PCR.

Nested PCR

Using a modified protocol for nested PCR we amplified 350 bp long amplicons specific for 18SSU rRNA gene of *Cryptosporidium* species.^{13,14}

The volume of the PCR reaction mixtures was, in both cases, 50 μ L, from which the DNA sample was 5 μ L. In these reactions, we used primers with a concentration of 0.2 μ M and 5 U Taq DNA polymerases (FIREPol).

The PCRs were run in a thermo cycler (XP Thermal Cycler Blocks) with an initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, $61/57^{\circ}\text{C}$ for 1 min, and 72°C for 2 min. A final elongation step at 72°C for 7 min was included for the complete extension of the amplified products.

Electrophoresis and sequencing

A secondary PCR product was evaluated by electrophoresis and visualized under UV light with 312 nm wavelength. Samples that were positive after sequencing were consequently compared to sequences stored in the GenBank in accordance with the genetic marker of 18 SSU rRNA gene.¹⁵

PCR products were directly sequenced in both directions. The sequences were aligned and completed using Chromas Pro Programme and compared to known sequences in the National Centre for Biotechnology Information GenBank database.

Phylogenetic analysis

The sequenced data were processed to form a sequence alignment for identifying similarities using MEGA6 software in subdirectory Align with CLUSTAL W option. Subsequently, the phylogenetic tree was constructed also with MEGA6 software using a Phylogeny menu and Maximum Likelihood method.

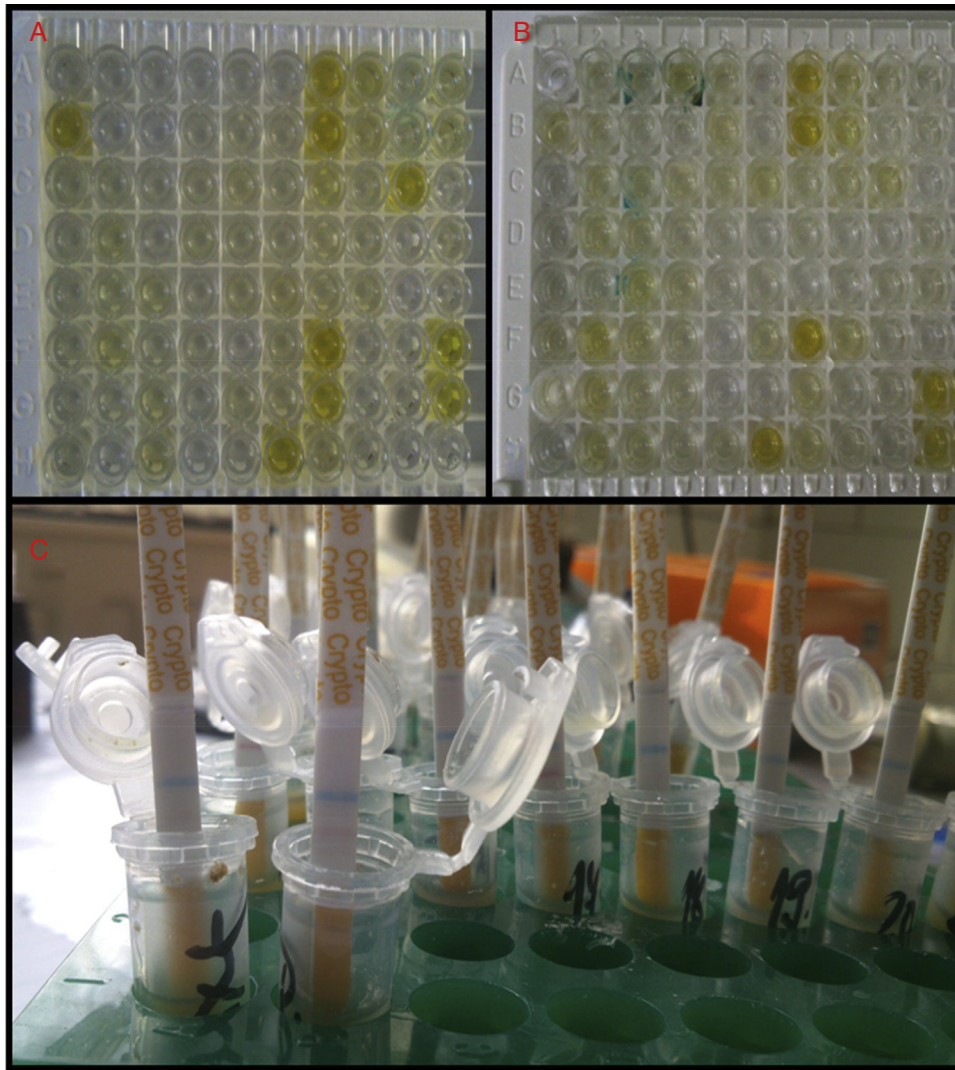


Fig. 1 – Comparison of three commercially available Copro-antigen tests. (A) RIDASCREEN[®] *Cryptosporidium* test [Enzyme Immunoassay (EIA)], (B) *Cryptosporidium* 2nd Generation [Enzyme Linked Immunosorbent Assay (ELISA)], (C) RIDA[®] QUICK *Cryptosporidium* [Immuno-chromatographic test (ICT)].

Immunology tests

EIA

A commercial Copro-antigen ELISA EIA kit [RIDASCREEN[®] *Cryptosporidium* (R-BIOPHARM-AG, Germany)] was used according to the manufacturer's instructions (Fig. 1). Briefly, 100 μ L of the liquid faecal or stool samples were diluted 1:10 with dilution buffer, mixed well and incubated at room temperature for 15 min. Then, 100 μ L of the supernatant from each sample was placed in a test well. Then, 100 μ L of the enzyme conjugate was added and plates were incubated at room temperature for 60 min. After washing five times with diluted washing buffer, the wells were incubated with 100 μ L substrate solution for 15 min before 50 μ L of stop reagent were added. Optical densities were determined at 450 and 620 nm using an Epoch ELISA reader (Biotek, Bad Friedrichshall). According to the cut-off value provided by the manufacturer (extinction for the negative control+0.15), samples were

considered positive if their extinction was more than 10% above the calculated cut-off value and questionable if it was between the cut-off and the cut-off plus 10%.

ELISA

Quantitative detection of *Cryptosporidium* antigen in stool samples was carried out by in vitro immunoassay using a commercial kit *Cryptosporidium* ELISA (Diagnostic Automation, INC, Calabasas, CA), (Fig. 1). In the case of a positive immunological reaction for the presence of *Cryptosporidium* antigen, the absorbance (450/630 nm, ELISA Reader Optys MR Thermo Labsystems) was read of 0.15 OD units and above. Absorbance reading less than 0.15 OD units indicated that the sample did not contain detectable levels of *Cryptosporidium* antigen.

ICT

The commercially available kit RIDA[®] QUICK *Cryptosporidium*/*Giardia* Combi (R-BIOPHARM AG) was used according

to the manufacturer's instructions (Fig. 1). Briefly, a 100- μ L aliquot from the liquid faecal or stool samples was diluted 1:10 with the dilution buffer, vortexed thoroughly and allowed to settle for 3 min. From the supernatant, 500 μ L aliquots were transferred to new test tubes. Test strips were immersed in the supernatant for 5–10 min. A sample positive for *Cryptosporidium* gives a blue band which appears along with a green control band.¹⁶

Ethical clearance

The study was approved by the institutional ethics committee of the University of Veterinary Medicine and Pharmacy in Košice, Slovakia.

Results

In our study, by analysing 79 samples of animal faeces using PCR and sequencing, we detected true positivity in 22 cases (27.84%), in which we detected 6 species – *C. parvum*, *C. bovis*, *C. scrofarum*, *C. suis*, *C. muris*, *C. andersoni* (Table 1).

Results of the three immunoassay were as follows: using EIA, ELISA and ICT to examine 35 samples from pigs (1st group), positive were 14 (40.0%), 7 (20.0%) and 0 (0.0%) samples, respectively. In calves (2nd group), there were positive 13 (38.23%), 13 (38.23%) and 5 (14.7%) samples, and in the lambs (3rd group) 0 (0.0%), 2 (20.0%) and 0 (0.0%) samples by EIA, ELISA and ICT, respectively. The prevalence of cryptosporidiosis in all faecal samples was 27 (34.17%), 22 (27.84%) and 5 (6.33%) by EIA, ELISA and ICT, respectively (Table 1). The prevalence detected by EIA (34.17%) and ELISA (27.84%) methods was about the same, but it was not a confirmation of a proven positivity in the same sample by several methods. Using PCR as a reference method, animal samples sensitivity (SE) of the EIA, ELISA and ICT was 63.6%, 40.9% and 22.7%, respectively, when questionable samples were considered positive, whereas specificity (SP) of EIA, ELISA, ICT was 75.9%, 78.9%, 100%, respectively. Positive predictive values (PPV) and negative predictive values (NPV) were different for all three tests (Table 2).

Phylogenetic tree for partial fragment of the 18S rRNA gene

For creating a phylogenetic tree, 21 sequences were identified, before used for immunology tests, by BLAST as *Cryptosporidium* spp. and 17 reference samples from NCBI, were used as the best matching species (Fig. 2). As an out-group we used reference sequences (NCBI) *Eimeria* sp. (JQ993661.1), *Eimeria tenella* (JX093900.1) and *Eimeria zuernii* (AY876923.1). Evolutionary branching of sequences in the phylogenetic tree also showed relationship between sequences identified as *C. parvum* and *C. bovis*, *C. scrofarum*, *C. suis*, *C. muris*, *C. andersoni*.

Discussion

In the recent years, the number of commercial diagnostic immunoassays for the presence of the antigen to *Cryptosporidium* spp. antibodies increased rapidly, focusing especially on speed, ease and sufficient sensitivity of testing.

However, these tests are used as screening tests, provide only quantitative results and are suitable only for detecting the presence/absence of infection in large groups of animals or humans.^{6,8} Weitzel et al.,¹⁷ Helmy et al.,¹⁶ and Uppal et al.,¹⁸ in their studies showed the inaccuracy of immunoassays and a higher detection rate of the pathogen by PCR methods. In our study, we confirmed the inaccuracy of immunoassays not only with regard to the percentage difference between the tests, but also the diversity of individual positive samples analyzed by each test. When using the PCR method and sequencing, we detected infection with *Cryptosporidium* spp. in 28.57% of samples not only in samples positive in at least one immunoassay but also in five samples where the presence of infection was not confirmed by any of the three Copro-antigen immunoassays. The prevalence detected by EIA (34.17%) and ELISA (27.84%) methods was nearly the same, but it was not a confirmation of a proven positivity in the same sample by several methods (Table 1 and Fig. 1). The sensitivity (SE) of animal samples detected with EIA, ELISA and ICT was 63.6%, 40.9% and 22.7%, respectively, when questionable samples were considered positive, whereas specificity (SP) of EIA, ELISA and ICT was 75.9%, 78.9%, 100%, respectively (Table 2). Despite the fact that the producers of the mentioned immunoassays declare 100% specificity and 100% sensitivity, the differences observed in our study not only between individual assays but also between different groups indicate controversial reliability and reproducibility of tests. Therefore, the results of serological tests that are used to detect antigens of *Cryptosporidium* spp. in the faeces, either positive or negative, obtained by a single diagnostic method are not reliable and appear insufficient, particularly with respect to patients from life-threatening risk groups (immunosuppressed patients).

Possible explanation of controversial results of Copro-antigen immunological tests for *Cryptosporidium* spp. detection is that not all commercially produced antibodies are able to recognize all *Cryptosporidium* spp. oocysts antigens of individual species, although all soluble and insoluble antigens are detected by EIA tests. Antibodies in the respective kits are not responding or respond only weakly to antigens of some species that are genetically distant from species *C. parvum* and *C. hominis*, which serve as a basis for production of these tests.^{19,20} Our phylogenetic analysis confirmed genetic variability of individual species and by that also probability that each *Cryptosporidium* spp. can produce various Copro-antigens which are not inevitably detectable by immunological tests, as these tests are commercially produced using antigens of species *C. parvum* and *C. hominis*.^{19,20} Species *C. parvum* and *C. hominis* are also detectable by means of common genes specific for them (e.g. GP 60), which compared to other *Cryptosporidium* spp. enable not only their identification but also genotyping and thus indicate their genetic variability within the species. The immunological tests use *Cryptosporidium* spp. that occur most frequently in individual animals, thus exhibiting a probable host specificity for the given animal species. In our study, positivity was detected by all three immunological tests only for one species, namely *C. parvum*, but only in three samples, although we tested 10 positive samples of faeces for the presence of *C. parvum* (Table 1). The primary identification of non-specific species *C. muris* and *C. andersoni* in pigs, different localization on phylogenetic tree and inability of detection

Table 1 – Comparison of positive results of different diagnostic tests for the detection of *Cryptosporidium* in faecal samples.

Species	EIA	ELISA	ICT	PCR	Identified species
Pigs (n = 35)	–	–	–	+	<i>C. muris</i>
	+	–	–	+	<i>C. muris</i>
	+	–	–	+	<i>C. scrofarum</i>
	–	–	–	+	<i>C. scrofarum</i>
	+	–	–	–	
	+	+	–	–	
	+	+	–	–	
	+	–	–	–	
	–	–	–	+	<i>C. suis</i>
	+	–	–	+	<i>C. suis</i>
	–	+	–	+	<i>C. scrofarum</i>
	+	–	–	+	<i>C. scrofarum</i>
	+	–	–	–	
	–	+	–	–	
	+	–	–	–	
–	+	–	+	<i>C. scrofarum</i>	
+	+	–	–		
+	–	–	+	<i>C. andersoni</i>	
No. of positive samples	14	7	0	10	
Calves (n = 34)	+	–	–	+	<i>C. bovis</i>
	–	–	–	+	<i>C. bovis</i>
	–	+	–	–	
	+	+	–	+	<i>C. parvum</i>
	+	+	+	+	<i>C. parvum</i>
	+	+	+	+	<i>C. parvum</i>
	–	+	–	–	
	+	+	–	+	<i>C. parvum</i>
	+	+	+	+	<i>C. parvum</i>
	+	+	+	+	<i>C. parvum</i>
	+	+	–	–	
	+	+	–	–	
	+	+	–	–	
	–	–	–	+	<i>C. parvum</i>
	–	+	–	–	
+	–	–	+	<i>C. parvum</i>	
No. of positive samples	13	13	5	12	
Lambs (n = 10)	–	+	–	–	
No. of positive samples	0	2	0	0	
∑ Positive animals/%	27/34.17	22/27.84	5/6.33	22/27.84	

by immunological tests also indicate the potential of *C. muris* and *C. andersoni* to produce different Copro-antigens.

Another possibility is the occurrence of antigen subtypes and nonexistence of surface antigens, where specific glycoproteins necessary for detection are not expressed but are bound to unspecific groups (e.g. hydroxyl groups, basic sugars) due to reorganization and modification of gene regions, but not mutations. *Babesia* and *Plasmodium* have many antigen subtypes, where modification of gene region is evolutionary due to protection of the given pathogen. Antigen variations form when pathogen withstands host immunity through sequence modification of antigens located on erythrocytes

surface.²¹ Blake et al.²² pointed at antigen variability and genome diversity of pathogens from phylum *Apicomplexa* in their study, which examined similarities between genera *Plasmodium*, *Toxoplasma* and *Eimeria*, but also antigen variability of individual species from the same genus.

Serological tests are an appropriate diagnostic method in numerous clinical groups (e.g. animal herd) for determination of the presence of a pathogen, because of its speed, sufficient sensitivity and affordability. However, one should keep in mind that immunological tests are not capable of detecting every Copro-antigen of each of more than 30 species and many genotypes identified up to 2016. The species *C.*

Table 2 – Sensitivity (SE/%), specificity (SP/%), positive predictive values (PPV/%), and negative predictive values (NPV/%) of tests for detection *Cryptosporidium* spp. in stool samples.

		EIA	ELISA	ICT
Pigs	SE	35.7	20.0	0
	SP	64.0	80.0	100.0
	PPV	35.7	28.5	0
	NPV	76.2	71.4	71.4
Calves	SE	75.0	58.3	41.6
	SP	81.8	72.7	100.0
	PPV	69.2	53.8	100.0
	NPV	85.7	76.2	75.8
Lambs	SE	0	0	0
	SP	100.0	80.0	100.0
	PPV	0	0	0
	NPV	100.0	100.0	100.0
∑ Animals	SE	63.6	40.9	22.7
	SP	75.9	78.9	100.0
	PPV	51.8	40.9	100.0
	NPV	85.4	78.9	77.0

scrofarum, *C. suis* and *C. andersoni* identified in our study have been identified also in humans.^{23,24} The low reliability of immunological tests, particularly when identifying infection in one patient, necessitates to use for accurate diagnosis at least two, optimally three different diagnostic methods (microscopy, immunology and molecular methods). Molecular methods, including the polymerase chain reaction

(PCR) are considered reference methods not only for detection of cryptosporidiosis infection, but also for identification individual *Cryptosporidium* spp. and genotyping. And it is exactly the identification and genotyping of individual species by molecular methods that confirms variability among individual species and thus also different genotype and phenotype manifestations.^{21,22}

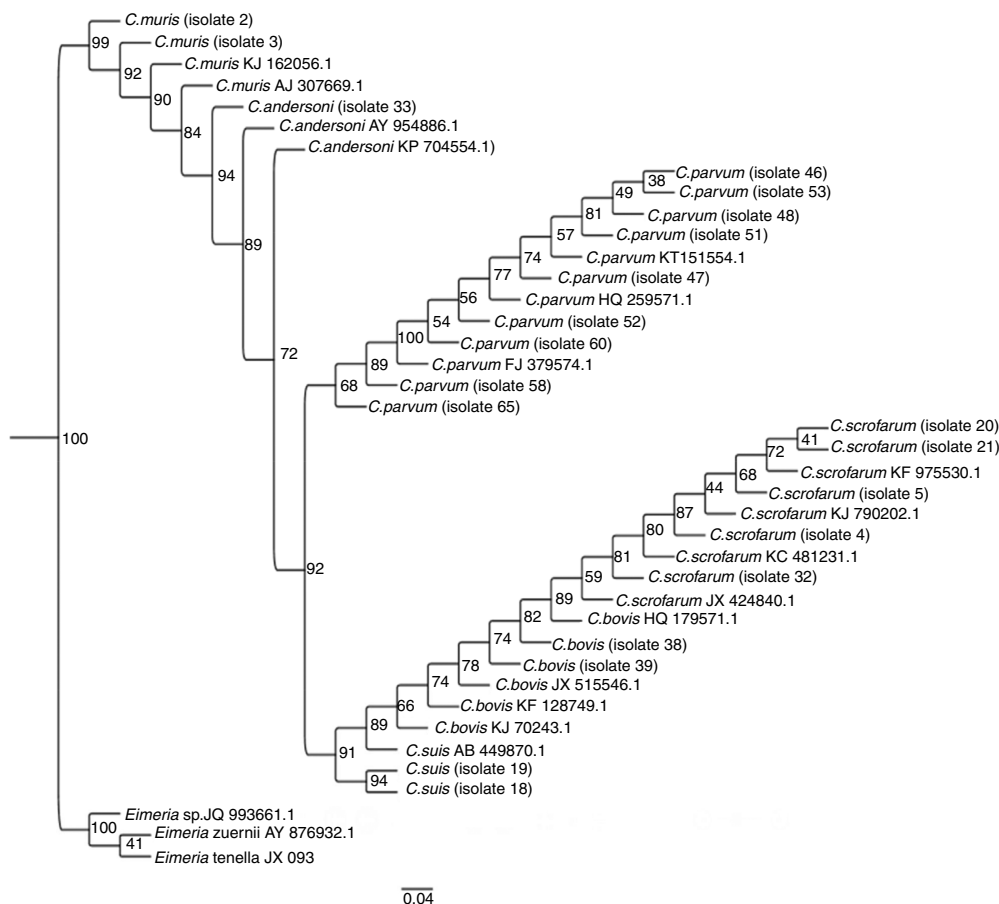


Fig. 2 – Evolutionary relationships among species *C. parvum* and *C. muris*, *C. andersoni*, *C. suis*, *C. scrofarum*, *C. bovis* inferred from a partial fragment of the 18S rRNA gene.

Conflicts of interest

The authors declare no conflicts of interest.

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