

Intestinal Parasites of Buffalo Calves from Romania: Molecular Characterization of *Cryptosporidium* spp. and *Giardia Duodenalis*, and the first Report of *Eimeria Bareillyi*

Diana Ancuța Bărburaș

University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca

Vasile Cozma

University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca

Angela Monica Ionică

University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca

Ibrahim Abbas

Mansoura University

Remus Bărburaș

S.C. Medivet Bărburaș S.R.L, Românași, nr. 299, Sălaj

Viorica Mircean

University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca

Gianluca D'Amico

University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca

Jitender P Dubey

5United States Department of Agriculture, Agricultural Research Service, Beltsville Agricultural Research Center, Animal Parasitic Disease Laboratory, Building 1001, BARC-East, Beltsville, MD 20705-23

Adriana Gyorke (✉ adriana.gyorke@usamvcluj.ro)

University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca

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Abstract

Buffaloes represent an important economic resource for several regions of the world including Romania; however, no reports on parasitic infections in buffaloes from Romania are available. In the present study, we examined for the gastrointestinal parasites 104 fecal samples bimonthly collected from 38 buffalo calves (2–11 weeks old) from household rearing systems in Romania. All samples were tested using the saturated salt flotation, McMaster and modified Ziehl-Nielsen staining methods. PCR coupled with isolates sequencing methods were used to identify the *Giardia duodenalis* assemblages and *Cryptosporidium* species. Overall, 33 out of 38 examined buffalo calves were infected with different gastrointestinal parasites; 16 had single infections and 17 had mixed infections with 2 or 3 parasites. *Eimeria* species (32/38; 84.2%) was the most prevalent parasite; 8 species were identified according to the oocyst morphology including the pathogenic *E. bareillyi* which detected for the first time in buffaloes from Romania. *Toxocara vitulorum* (11/38; 36.8%) and *Strongyloides papillosus* (6/38; 15.8%) were also detected. *Cryptosporidium* spp. were found in 4 (10.5%) buffalo calves; 2 of them were molecularly identified as *C. ryanae* and another one was clustered in the same clade with *C. ryanae*, *C. bovis*, and *C. xiaoi*. *Giardia duodenalis* assemblage E was also molecularly detected in a single (2.6%) buffalo calf. The presence of other buffaloes in the same barn was identified as a risk factor for infection with *T. vitulorum*. Our results indicate extensive parasitic infections in buffalo calves from Northwestern Romania and underline the necessity of prophylactic treatments for *T. vitulorum* and *E. bareillyi*.

Introduction

The worldwide population of water buffaloes (*Bubalus bubalis*) has been estimated as 206 million in 2018, with most populations being found in Asia (97%); buffaloes represent a major contribution to the economy of these regions ¹. In Europe, buffaloes are mainly found in Italy, Romania, Bulgaria, Greece, Germany, United Kingdom, Macedonia, and Albania ².

Parasitic infections can cause serious production losses in this animal species ³. Buffaloes are infected with several parasites and some of them have a zoonotic nature; for example, the ubiquitous protozoan *Cryptosporidium*, which cause serious disease in humans and animals. Three *Cryptosporidium* species are detected in buffaloes: *C. parvum*, *C. ryanae* and *C. bovis* ^{4–7}. *C. parvum* circulates among humans and different animals, and the infection is generated by ingestion of the oocysts which are dispersed widely in the environment and can resist harsh environmental conditions ⁸. Molecular data has revealed that in many instances, ruminants represented the major sources of *C. parvum* infection outbreaks in human ⁹.

Another example is the coccidiosis which is a major disease of livestock, including buffaloes, and caused by the protozoan *Eimeria*. Although *Eimeria* species are in general host-specific, cattle and buffaloes have been reported to share many species. However, *E. bareillyi* is specific to buffaloes and is also the most pathogenic ¹⁰.

Reports on the parasitic infections in buffaloes worldwide are few, and no reports from Romania are available. The objective of the present study was to determine the prevalence and species composition of different gastrointestinal (GIT) parasites infecting buffalo calves kept in household rearing systems in Romania. The revealed data would be important epidemiologically for the implementation of effective surveillance and control programs.

Results

Thirty-three (86.8%) out of 38 examined buffalo calves were infected with at least one GIT parasite. Single (42.1%), dual (42.1%) and triple (2.6%) patterns of infection were noted. Five parasitic genera were detected; *Eimeria* spp. was the highly prevalent (32/38; 84.2%) followed by *T. vitulorum* (11/38; 36.8%), *S. papillosus* (6/38; 15.8%), *Cryptosporidium* spp. (4/38; 10.5%), and *G. duodenalis* (1/38; 2.6%) (Table 3).

Eight *Eimeria* species were identified: *E. bareillyi*, *E. zuernii*, *E. cylindrica*, *E. ellipsoidalis*, *E. auburnensis*, *E. canadensis*, *E. subspherica* and *E. bovis*. Oocysts of all species, but not *E. subspherica*, were noticed in feces of 2–3 weeks old calves (55.6% were infected) and the prevalence increased with the age (84.6% in 10–11 weeks old). *Eimeria bareillyi* was highly prevalent in 2–3 (36.1%) and 6–7 (76.3%) weeks age groups (Table 3). The intensity of infection was 8,715–1,439,941 OPG, being the highest in 2-5-weeks old calves and decreased with the age (Table 4).

T. vitulorum eggs were identified in all age groups with a prevalence ranging from 11.1% in 2-3-weeks old buffalo calves to 23.1% in 10-11-weeks old buffalo calves (Table 3). The EPG value decreased with the age from 70,415 to 6,359 (Table 4), and the presence of adult buffaloes in the same barn with buffalo calves was identified as a risk factor for *T. vitulorum* infections (Table 5).

Strongyloides papillosus eggs were detected starting with 4-5-weeks of age and the prevalence increased with the age from 3.7 to 23.1% (Table 3), with an estimated EPG 444 on average.

Oocysts of *Cryptosporidium* spp. were detected in feces of 4 calves (3–4 weeks old). DNA of 3 isolates was successfully amplified and sequenced; 2 isolates were defined, according to the BLAST search, as *C. ryanae* (acc. no. MW289064, MW289065) and clustered with various *C. ryanae* isolates from dairy calves worldwide particularly those from Asia (China and India). The third isolate (acc. no. MW289066) was included in the same clade with sequences of *C. ryanae*, *C. bovis*, and *C. xiaoi*, but in a separate branch (Fig. 1).

Giardia duodenalis was molecularly identified in feces of a 9-weeks old buffalo calf. The revealed nucleotide sequence was identical to the isolate AB692776 from sheep in Iran, which belongs to assemblage E (acc. no. MW289556).

Discussion

During the present study, the prevalence of intestinal parasites was evaluated in buffalo calves aged between 2 and 11 weeks in northwestern Romania. Three protozoan parasites (*Eimeria* spp., *Cryptosporidium* spp., and *G. duodenalis*) and two nematodes (*T. vitulorum* and *S. papillosus*) were identified. These GIT parasites are the most common found in buffalo calves worldwide ^{11,12}.

In the present study, *Eimeria* spp. was the most prevalent GIT parasite and detected in 84.2% of buffalo calves. This ubiquitous protozoan has a worldwide dispersal in water buffaloes which could be infected with at least 12 *Eimeria* species, of them 11 are of cattle origin ¹³. So far, *E. bareillyi* is the only buffalo-specific species, non-transmissible to cattle, and is highly pathogenic to young calves ¹⁴. This is the first report of *E. bareillyi* oocysts in buffalo calves from Romania; however, infections were subclinical, and the oocysts were detected in all age groups. *E. bareillyi* infections have been reported worldwide (Dubey, 2018) including 2 reports from Europe; in Italy ^{15,16} and the Netherlands ¹⁷. The prepatent period is 12–15 days after experimental infection with 350000-15000000 *E. bareillyi* oocysts ¹⁴. In natural infections, oocysts were detected at 13 days of age ¹⁸; however, oocysts from other species (e.g. *E. bovis*, *E. ellipsoidalis*, *E. auburnensis* and *E. zuernii*) were noticed as early as 2–7 days of age ¹⁹. Therefore, a study on buffalo calves from *E. bareillyi*-infected farm and involving daily examination of fecal samples from the 1st day of birth is required to detect whether *E. bareillyi* oocysts are excreted in the first week of life.

The second most found parasite in the present study is *T. vitulorum* (36.8%), which is frequently found in tropical and sub-tropical regions ^{20,21}, and less often in temperate climates as in Europe. This parasite can cause significant morbidity and mortalities in calves. We noticed *T. vitulorum* eggs, but not the mature worms, in feces of the examined calves of all age groups. *T. vitulorum* larvae are passed in great numbers in the buffalo cows colostrum 2–5 days post-calving, worms are matured in the intestine of the calves by 10 days of age and eggs are passed in feces approximately at the 3rd week of life and then adult worms are expelled from the intestine by the 5th month of age ^{22,23}. In Europe, this nematode parasite was reported in the latest years at the farm level in cattle ^{24–29} and bisons ³⁰. In western Romania, the parasite was detected in 0.3% of 303 fecal samples from cattle ³¹.

Additionally, *S. papillosus* eggs were found in 15.8% of the examined buffalo-calves in the present study, and the prevalence increased with the age. A lower prevalence (3.1%) was detected in water buffaloes (adults, heifer/steers and calves) from Italy ³². However, higher values have been reported worldwide: 28.5% in India (Jyoti et al., 2014); 59.4% in Mexico (Ojeda-Robertos et al., 2017) and 87% in 3 months old calves in Sri Lanka ^{33–35}. Despite *Strongyloides* species infects all age groups, clinical signs such as diarrhea and malnutrition are frequently seen in young animals ³⁶. Sudden deaths were reported in naturally and experimentally infected calves and the dead calves harbored an EPG value between 52000 and 411000 ³⁷. *Strongyloides*-infected calves from our study presented low EPG (under 1000). Considering the high pathogenicity of the parasite, calves with high EPG (> 10000) should be treated ³⁶.

Our results demonstrate *Cryptosporidium* infections in 4 (10.5%) buffalo calves; this prevalence is comparable to earlier studies worldwide^{4,5,7}. However, our prevalence is much lower than that from buffalo-calves (2–4 months) from Egypt (40.0%) and Brazil (54.1%)^{6,38}. Compared to cattle (25.19–28.52%) from the country of study (Romania), the obtained prevalence in buffalo calves is lower^{39,40}. DNA from 3 of the four *Cryptosporidium* isolates was successfully amplified and sequenced, and the obtained sequences revealed *C. ryanae* infection in 2 samples, whereas the 3rd isolate was unidentified but related genetically to the bovine *C. ryanae* and *C. bovis* as well as the ovine *C. xiaoi*. *C. ryanae* is the most common *Cryptosporidium* species reported worldwide in buffaloes^{41,42}, but also *C. parvum*, and *C. bovis*^{41–44}. In Romania, *C. parvum* was reported in cattle, and as the predominant species in lambs^{40,45,46}. Our results cannot deny the role of buffaloes in the transmission cycle of *C. parvum* due to the limited number of tested samples and sequenced isolates.

Moreover, a single sample was positive for *G. duodenalis* infection, and sequence analysis revealed the occurrence of the assemblage E. This assemblage is non zoonotic and commonly circulates among different farm animals including cattle, sheep, goat and horses⁴⁷, and has been detected together with the assemblage A in buffaloes from Italy (Caccio et al., 2007), Australia^{4,48,49} Egypt (Helmy et al., 2013).

Generally, buffaloes are considered robust species and therefore more resistant to diseases compared with cattle. However, this study pointed out that pre-weaned buffalo-calves are exposed to early infection with pathogenic *E. bareillyi*, and other *Eimeria* species, and to the infection with *T. vitulorum*. Moreover, the infection risk for *T. vitulorum* increased when in the same barn were more than two buffaloes. Multiple infections, and currently parasitizing the same region of intestine, can adversely affect the growth of buffaloes. These data pointed out the need for early antiparasitic treatments for coccidiosis and toxocarosis in buffalo-calves. Buffalo-calves are not a source of zoonotic *C. parvum* and *G. duodenalis* in our area.

Materials And Methods

Samples collection

Rectal fecal samples from 38 buffalo calves were collected during Spring from March to June 2017. Buffaloes were reared in 4 villages (Românași, Păușa, Poarta Sălajului and Chichișa) from Sălaj County, northwestern Romania. One buffalo calf was sampled per household. During this period, animals remained in the barns. Each buffalo calf was sampled ≤ 5 times every 14 days, starting from the age of 2–3 weeks, until 10–11 weeks. In total, 104 fecal samples were collected because not all the buffalo calves remained till the end in the study. Each of the sampled animals was identified by a unique code, and data regarding age, sex, origin and the presence of other buffaloes or cattle in their enclosures was recorded.

Sample analysis

Fecal samples were examined using sodium chloride (specific gravity 1.28) flotation method. The revealed cysts, oocysts and eggs were identified according to their morphological characters^{10,13,50}, using an optical microscope (Olympus BX61, Japan). Positive samples were further tested using the McMaster technique in order to determine the number of oocysts/eggs per gram (OPG/EPG) of fecal sample. Ziehl-Nielsen modified staining was used to confirm the identity of *Cryptosporidium* spp. oocysts. Microphotographs of oocysts were taken using an optical microscope (Olympus BX61, Japan) connected to a DP72 camera (Olympus Corporation, Japan), and the measurements were determined with Cell[^]F software (Olympus Corporation, Japan). Different *Eimeria* species were identified based on oocyst morphology, as described previously^{10,13}.

Molecular analysis

All fecal samples were molecularly tested to detect *G. duodenalis* and *Cryptosporidium* spp. DNA, and further positive samples were sequenced to identify assemblages and species, respectively. DNA was extracted from each sample, using a commercial kit (Isolate II Fecal DNA Kit, Bioline, UK), according to the manufacturer's instructions. The harvested DNA was stored at -20 °C until further processing.

Giardia duodenalis 432 bp fragment of the glutamate dehydrogenase gene (*gdh*) and *Cryptosporidium* 638 bp fragment of the SSU rRNA gene were amplified by means of nested PCR reactions^{51,52}. The primers and reaction conditions are listed in Tables 1 and 2. Reactions were conducted using a C100 Thermal Cycler (Bio-Rad, Hercules, USA) in a final volume of 25 µl which consisted of 12.5 µl MyTaq Red HS Mix 2x master mix (Bioline, UK), 25 pmol of primers, and 4 µl of genomic DNA which was replaced in the second round by 4 µl of the first-round product.

PCR products were visualized by electrophoresis on 1.5% agarose gel stained with SYBR® Safe DNA Gel Stain (Thermo Fisher Scientific Inc., USA) and their molecular weight was assessed by comparison to a molecular marker (O'GeneRuler™ 100 bp DNA Ladder, Thermo Fisher Scientific Inc., USA).

PCR products were purified using a commercial kit (FavorPrep GEL/PCR Purification Mini Kit, Favorgen Biotech Corp., Taiwan) and commercially sequenced (Macrogen Europe B.V., Netherlands). The revealed sequences were compared to other sequences available in the GenBank™ database, using the Basic Local Alignment Search Tool (BLAST) analysis. The phylogenetic analysis was conducted using MEGA X software, and the evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Jukes-Cantor method. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1).

Statistical analysis

The frequency, prevalence and its 95% confidence interval (95% CI) were calculated for each identified parasite. These were performed overall, and according to age group. Five age groups were established: 2–3 weeks, 4–5 weeks, 6–7 weeks, 8–9 weeks, and 10–11 weeks.

The average and standard error were calculated for OPG and EPG. Before further processing, the distribution data was assessed by D'Agostino-Pearson's normality test. If not normally distributed, the data were transformed by logarithm in base 10. The ANOVA repeated measures analysis of variance test was used to identify statistically significant differences among age groups.

Risk factors, such as the presence of young and adult buffaloes, and the presence of cattle in the same barn were evaluated by chi-square test. A value of $p < 0.05$ was considered statistically significant. Statistical analysis was performed with EpiInfo 3.5.1 (CDC, USA) and MedCalc Statistical Software 19.0.4. (MedCalc Software Ltd, Ostend, Belgium).

Declarations

Author contributions:

DAB, AMI: Conceptualization, Formal analysis, Investigation, Methodology, Writing - original draft; VC: Conceptualization, Supervision, Validation; IA: Data curation, Formal analysis, Validation, Writing - review & editing; RB, VM, GD: Methodology; DJP: Writing - review & editing; AG: Conceptualization, Data curation, Formal analysis, Resources, Supervision, Validation, Visualization, Writing - review & editing.

Competing interests.

The authors declare no competing interests.

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Tables

Table 1
Primer sequences used for the nested PCR

Species	Oligonucleotide primer	Oligonucleotide sequence	Size of the amplified amplicon (pb)
<i>Cryptosporidium</i> spp.	CrSSU-1	GATTAAGCCATGCATGTCTAA	638
	CrSSU-2	TTCCATGCTGGAGTATTCAAG	
	CrSSU-3	CAGTTATAGTTTACTTGATAA	
	CrSSU-4	CCTGCTTTAAGCACTCTAATTTTC	
<i>Giardia duodenalis</i>	GDHeF	TCA ACG TYA AYC GYG GYT TCC GT	432
	GDHiR	GTT RTC CTT GCA CAT CTC C	
	GDHiF	CAG TAC AAC TCY GCT CTC GG	
	GDHiR	GTT RTC CTT GCA CAT CTC C	

Table 2
Reaction conditions in the employed nested PCRs

		nPCR ¹ (<i>Cryptosporidium</i> spp.)	nPCR ² (<i>Giardia duodenalis</i>)
First-Round Amplification^a	Initial denaturation	95°C, 5 min	95°C, 2 min
	Denaturation	95°C, 30s	95°C, 30s
	Annealing	53°C, 50s	55°C, 20s
	Elongation	72°C, 1 min	72°C, 45s
	Final extension	72°C, 5 min.	72°C, 7 min.
Nested Amplification^b	Initial denaturation	95°C, 5 min	95°C, 2 min
	Denaturation	95°C, 30 s	95°C, 20s
	Annealing	56°C, 50 s	53°C, 10 s
	Elongation	72°C, 1 min	72°C, 1 min
	Final extension	72°C, 5 min	72°C, 5 min
^{1a} 35 cycles; ^{1b} 40 cycles; ^{2a} 55 cycles; ^{2b} 40 cycles.			

Table 3
 Frequency and prevalence [nP/nT (%)] of the revealed GIT parasites in buffalo calves of different age groups

	2–3 weeks	4–5 weeks	6–7 weeks	8–9 weeks	10–11 weeks	Total
G. duodenalis	0/18	0/27	0/24	1/18 (5.6)	0/13	1/38 (2.6)
Cryptosporidium spp.	1/18 (5.6)	3 ^a /27 (11.1)	0/24	0/18	0/13	4 ^b /38 (10.5)
Total Eimeria spp.	10/18 (55.6)	23/27 (85.2)	22/24 (91.6)	18/18 (100)	11/13 (84.6)	32/38 (84.2) ^{***}
E. bareillyi	22/61 (36.1)	3/37 (8.1)	29/38 (76.3)	7/35 (20.0)	14/61 (23.0)	75/232 (32.3)
E. zuernii	16/61 (26.2)	13/37 (35.1)	4/38 (10.5)	8/35 (22.9)	11/61 (18.0)	52/232 (22.4)
E. cylindrica	12/61 (19.7)	7/37 (18.9)	3/38 (7.9)	6/35 (17.1)	6/61 (9.8)	34/232 (14.7)
E. ellipsoidalis	3/61 (4.9)	12/37 (32.4)	1/38 (2.6)	1/35 (2.9)	16/61 (26.2)	33/232 (14.2)
E. auburnensis	5/61 (8.2)	1/37 (2.7)	0/38	7/35 (20.0)	6/61 (9.8)	19/232 (8.2)
E. canadensis	1/61 (1.6)	1/37 (2.7)	1 (2.6)	6/35 (17.1)	0/61	9/232 (3.9)
E. subspherica	0/61	0/37	0/38	0/35	6/61 (9.8)	6/232 (2.6)
E. bovis	2/61 (3.3)	0/37	0/38	0/35	2/61 (3.3)	4/232 (1.7)
T. vitulorum	2/18 (11.1)	4/27 (14.8)	4/24 (16.6)	4/18 (22.2)	3/13 (23.1)	11/38 (36.8)
S. papillosus	0/18	1/27 (3.7)	1/24 (4.2)	1/18 (5.6)	3/13 (23.1)	6/38 (15.8)
Single infection	9/18 (50.0)	15/27 (55.6)	17/24 (70.8)	13/18 (72.2)	7/13 (53.8)	16/38 (42.1)
Mixed infection^c	2/18 (11.1)	8/27 (29.6)	5/24 (20.8)	5 ^d /18 (27.8)	5/13 (38.5)	17/38 (44.7)
Total	11/18 (61.1)	23/27 (85.2)	22/24 (91.6)	18/18 (100)	12/13 (92.3)	33/38 (86.8)

Table 4

OPG/EPG (mean± std. err.) values for the revealed GIT parasites in buffalo calves of different age groups

	2–3 weeks	4–5 weeks	6–7 weeks	8–9 weeks	10–11 weeks	Average
Eimeria spp.	83,914 ± 48,411	1,439,941 ± 1,350,781	149,988 ± 96,959	8,715 ± 3,289	81,214 ± 59,937	417,156 ± 338,143
T. vitulorum	70,415 ± 21,503	26,678 ± 6,420	23,192 ± 13,348	9,186 ± 3,590	6,359 ± 2,934	26,510 ± 7,085
S. papillosus	-	0	0	0	444 ± 82	444 ± 82

Table 5

Risk factors for infection with *T. vitulorum* in buffalo calves

	Frequency (prevalence)	OR	95% CI OR	<i>P</i>
Young buffaloes				
1–2 animals (<i>n</i> = 24)	2 (12.5)	Ref.		
3–4 animals (<i>n</i> = 14)	9 (91.7)	19.80	3.22–121.47	0.001
Adult buffaloes				
1–2 animals (<i>n</i> = 22)	2 (13.6)	Ref.		
3–5 animals (<i>n</i> = 16)	9 (68.8)	12.86	2.22–74.54	0.004
Cattle				
No (<i>n</i> = 29)	10 (44.8)	Ref.		
Yes (<i>n</i> = 9)	1 (11.1)	0.24	0.03–2.18	0.20

Figures

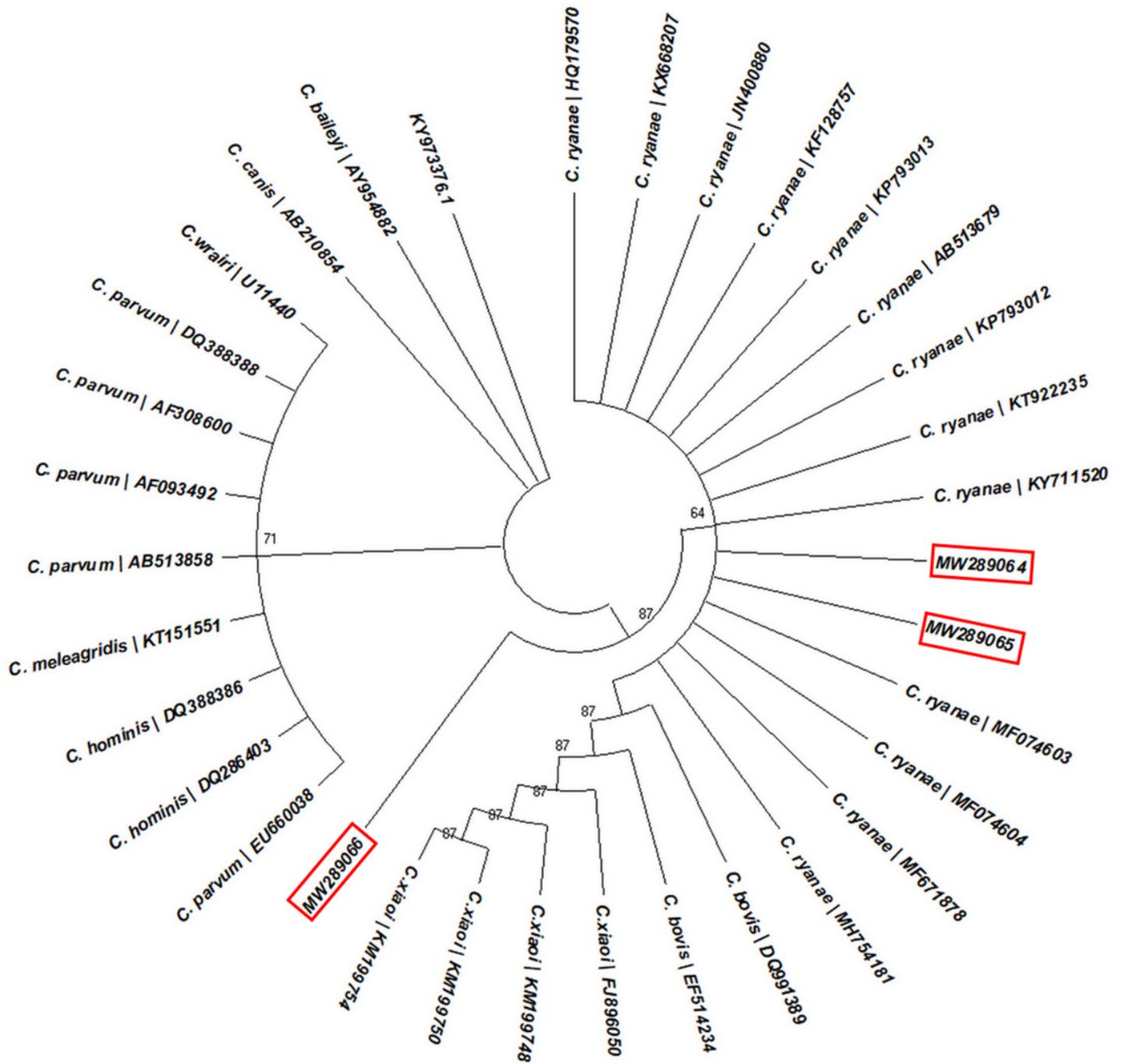


Figure 1

Bootstrap consensus tree for *Cryptosporidium* spp.. Branches corresponding to partitions reproduced in less than 50% of 1000 bootstrap replicates are collapsed.