

Research Article

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Intestinal parasites of buffalo calves from Romania: molecular characterisation of *Cryptosporidium* spp. and *Giardia duodenalis*, and the first report of *Eimeria bareillyi*

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Abstract: Buffaloes represent an important economic resource for several regions of the world including Romania. In the present study, we examined 104 faecal samples collected from 38 buffalo calves (2–11 weeks old) from household rearing systems in Romania for gastrointestinal parasites. All samples were tested using the saturated salt flotation, McMaster and modified Ziehl-Nielsen staining methods. PCR coupled with sequencing isolates were used to identify assemblages of *Giardia lamblia* (Kunstler, 1882) and species of *Cryptosporidium* Tyzzer, 1907. Overall, 33 out of 38 examined buffalo calves were infected with different gastrointestinal parasites: 16 had single infections and 17 had mixed infections with two or three parasites. Species of *Eimeria* Schneider, 1875 (32/38; 84%) were the most prevalent parasites; eight species were identified according to the oocyst morphology, including the pathogenic *E. bareillyi* (Gill, Chhabra et al., 1963) which was detected for the first time in buffaloes from Romania. The nematodes *Toxocara vitulorum* (Goeze, 1782) (11/38; 37%) and *Strongyloides papillosus* (Wedl, 1856) (6/38; 16%) were also detected. *Cryptosporidium* spp. were found in four (11%) buffalo calves; two of them were molecularly identified as *C. ryanae* Fayer, Santin et al., 2008, and another one clustered in the same clade with *C. ryanae*, *C. bovis* Fayer, Santin et al., 2005, and *C. xiaoi* Fayer et al., 2009. *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*.

Keywords: buffaloes, parasites, *Toxocara vitulorum*, *Eimeria bareillyi*, *Cryptosporidium*, *Giardia*

The worldwide population of water buffaloes *Bubalus bubalis* (Linnaeus) 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 (FAO 2020). In Europe, buffaloes are mainly found in Italy, Romania, Bulgaria, Greece, Germany, United Kingdom, Macedonia, and Albania (Borghese 2010).

Parasitic infections can cause serious production losses in this animal species (Thapa Shrestha et al. 2020). Buffaloes are infected with several parasites and some of them have a zoonotic nature, such as ubiquitous coccidia of the

genus *Cryptosporidium* Tyzzer, 1907, which cause serious disease in humans and animals. Three species of *Cryptosporidium* have been detected in buffaloes, namely *C. parvum* Tyzzer, 1912, *C. ryanae* Fayer, Santin et al., 2008 and *C. bovis* Fayer, Santin et al., 2005 (see Helmy et al. 2013, Aquino et al. 2015, Ma et al. 2015, Ibrahim et al. 2016). *Cryptosporidium 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 (Robertson et al. 2014). Molecular data have revealed that

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Table 1. Primers used for the nested PCR

Species	Oligonucleotide primer	Oligonucleotide sequence	Size of the amplified amplicon (bp)
<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	

ruminants represent the major sources of infection with *C. parvum* for humans (Thompson 2011).

Another example is coccidiosis which is a major disease of livestock, including buffaloes, and is caused by species of the genus *Eimeria* Schneider, 1875. Although species of *Eimeria* are in general host-specific, cattle and buffaloes have been reported to host many species. *Eimeria bareillyi* (Gill, Chhabra et Lall, 1963) is specific to buffaloes and is also the most pathogenic species (Dubey 2020).

Reports on the parasitic infections in buffaloes in our country are few. Therefore, 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 new data may be useful for the implementation of effective surveillance and control programs.

MATERIALS AND METHODS

Samples collection

Rectal faecal samples from 38 buffalo calves were collected from March to June 2017. Faecal samples were labelled, stored in plastic bags and sent during the day of collection in a cooling box to the laboratory. In the laboratory, the samples were stored in the refrigerator until the next day when the samples were analysed. Two hundred grams from each faecal sample were stored in the freezer for PCR analysis.

Samples were taken from buffaloes reared in four villages (Românaşi, Păuşa, Poarta Sălajului and Chichişa) 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. Sampling intervals were 14 days, starting from the age of 2–3 weeks, until 10–11 weeks. In total, 104 faecal samples were collected because not all calves were available until 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 were recorded.

Sample analysis

Faecal samples were examined using the sodium chloride (specific gravity 1.28) flotation method. Cysts, oocysts and eggs found were identified according to their morphological characters (Mircean et al. 2011, El-Alfy et al. 2019, Dubey 2020), using an optical microscope (Olympus BX61). Positive samples were further tested using the McMaster technique to determine the number of oocysts/eggs per gram (OPG/EPG) of a faecal sample. Ziehl-Nielsen modified staining was used to confirm the identity

of oocysts of *Cryptosporidium* spp. Microphotographs of oocysts were taken using an optical microscope (Olympus BX61) connected to a DP72 camera (Olympus Corporation, Tokyo, Japan), and the measurements were determined with Cell[^]F software (Olympus Corporation, Japan). Different species of *Eimeria* were identified based on oocyst morphology, as described previously (El-Alfy et al. 2019, Dubey 2020).

Molecular analysis

All faecal samples were molecularly tested to detect *Giardia duodenalis* (Stiles, 1902) and *Cryptosporidium* spp. DNA, and positive samples were further sequenced to identify assemblages and species, respectively. DNA was extracted from each sample, using a commercial kit (Isolate II Faecal DNA Kit, Bioline, London, 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 using nested PCR reactions (Lindergard et al. 2003, Read et al. 2004). 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, London, 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 visualised by electrophoresis on 1.5% agarose gel stained with SYBR[®] Safe DNA Gel Stain (Thermo Fisher Scientific Inc., Waltham, 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 (Favor-Prep GEL/PCR Purification Mini Kit, Favorgen Biotech Corp., Ping-Tung, Taiwan) and commercially sequenced (Macrogen Europe B.V., Amsterdam, 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 Maximum Likelihood method. The evolutionary distances were computed using the Jukes-Cantor model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.1650)).

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

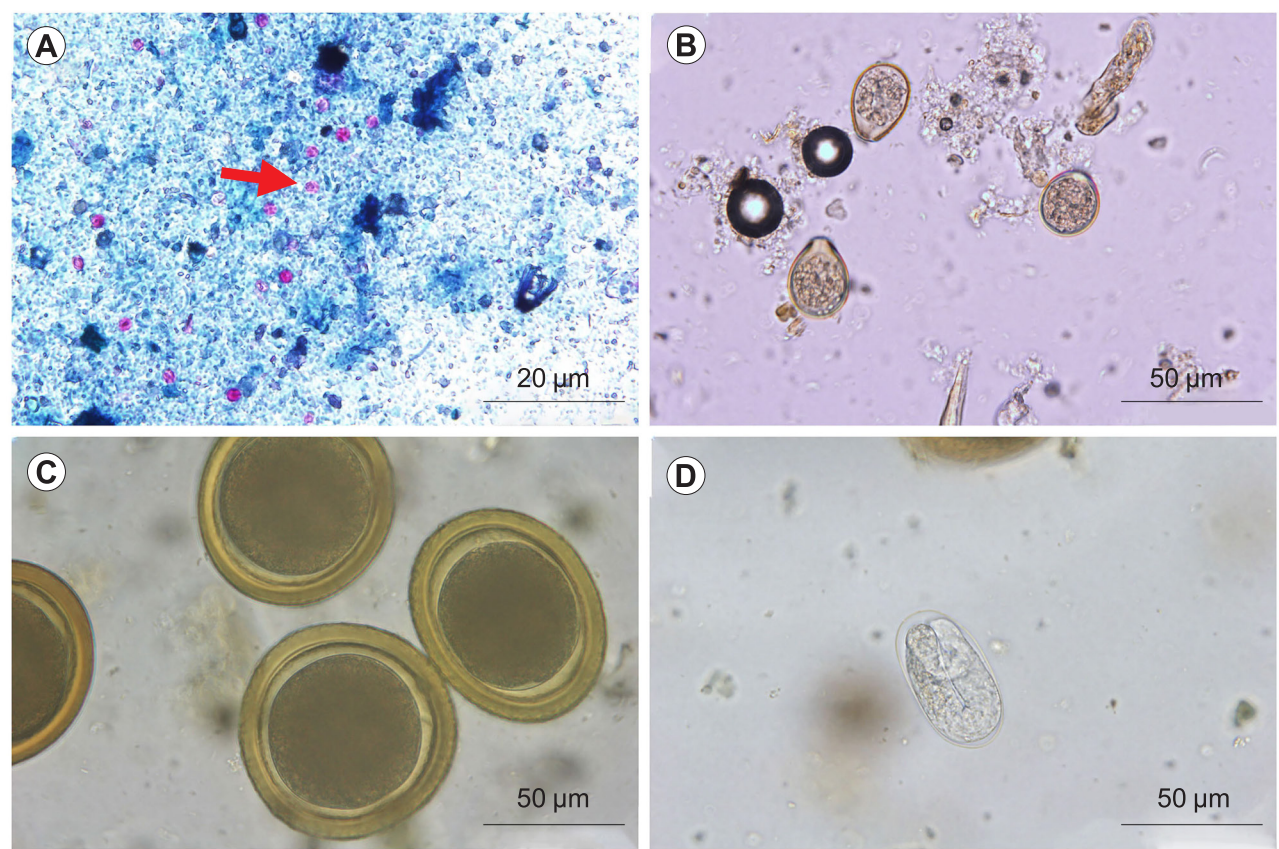


Fig. 1. Parasites of buffalo calves detected by coprology. **A** – oocysts (pink elements) of *Cryptosporidium* spp.; **B** – oocysts of *Eimeria* spp.; **C** – eggs of *Toxocara vitulorum* (Goeze, 1782); **D** – egg of *Strongyloides papillosus* (Wedl, 1856).

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, 30 s	95°C, 30 s
	Annealing	53°C, 50 s	55°C, 20 s
	Elongation	72°C, 1 min	72°C, 45 s
	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, 20 s
	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.

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).

RESULTS

Thirty-three (87%) out of 38 examined buffalo calves were infected with at least one GIT parasite. Single (42%), dual (42%), and triple (3%) infections were noted. Parasites of five genera were detected; species of *Eimeria* were most prevalent (32/38; 84%) followed by *Toxocara vitulorum* (Goeze, 1782) (11/38; 37%), *Strongyloides papillosus* (Wedl, 1856) (6/38; 16%), *Cryptosporidium* spp. (4/38; 11%), and *Giardia duodenalis* (1/38; 3%) (Fig. 1, Table 3).

Eight species of *Eimeria* were identified: *E. bareillyi*, *E. zuernii* Rivolta, 1878, *E. cylindrica* Wilson, 1931, *E. ellipsoidalis* Becker et Frye, 1929, *E. auburnensis* Christensen et Porter, 1939, *E. canadensis* Bruce, 1921, *E. subspherica* Christensen, 1941, and *E. bovis* (Fig. 2). Oocysts of all but one (*E. subspherica*) were found in faeces of 2–3 weeks old

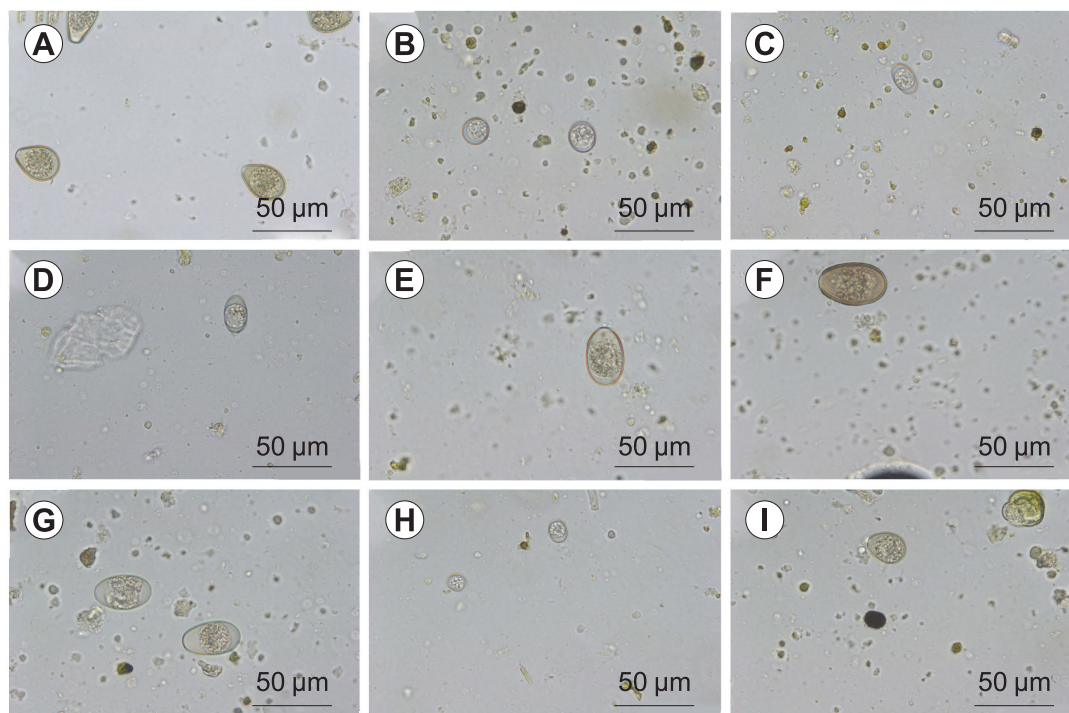


Fig. 2. Species of *Eimeria* Schneider, 1875 identified based on oocyst morphology in buffalo calves. **A** – *E. bareillyi*; **B** – *E. zuernii*; **C** – *E. cylindrica*; **D** – *E. ellipsoidalis*; **E** – *E. auburnensis*; **F** – *E. auburnensis*-like; **G** – *E. canadensis*; **H** – *E. subspherica*; **I** – *E. bovis*.

Table 3. Frequency and prevalence of gastrointestinal (GIT) parasites found in buffalo calves of different age groups.

	2–3 weeks	4–5 weeks	6–7 weeks	8–9 weeks	10–11 weeks	Total
<i>Giardia duodenalis</i>	0/18	0/27	0/24	1/18 (5.6)	0/13	1/38 (2.6)
<i>Cryptosporidium</i> spp.	1/18 (6%)	3/27 (11%)	0/24	0/18	0/13	4/38 (10.5)
Total <i>Eimeria</i> spp.	10/18 (55.6)	23/27 (85.2)	22/24 (92)	18/18 (100)	11/13 (85)	32/38 (84.2)***
<i>Eimeria bareillyi</i>	22/61 (36)	3/37 (8.1)	29/38 (76.3)	7/35 (20.0)	14/61 (23.0)	75/232 (32.3)
<i>E. zuernii</i>	16/61 (26)	13/37 (35.1)	4/38 (10.5)	8/35 (22.9)	11/61 (18.0)	52/232 (22.4)
<i>E. cylindrica</i>	12/61 (20)	7/37 (18.9)	3/38 (7.9)	6/35 (17.1)	6/61 (9.8)	34/232 (14.7)
<i>E. ellipsoidalis</i>	3/61 (4.9)	12/37 (32.4)	1/38 (2.6)	1/35 (2.9)	16/61 (26.2)	33/232 (14.2)
<i>E. auburnensis</i>	5/61 (8.2)	1/37 (2.7)	0/38	7/35 (20.0)	6/61 (9.8)	19/232 (8.2)
<i>E. canadensis</i>	1/61 (1.6)	1/37 (2.7)	1 (2.6)	6/35 (17.1)	0/61	9/232 (3.9)
<i>E. subspherica</i>	0/61	0/37	0/38	0/35	6/61 (9.8)	6/232 (2.6)
<i>E. bovis</i>	2/61 (3.3)	0/37	0/38	0/35	2/61 (3.3)	4/232 (1.7)
<i>Toxocara vitulorum</i>	2/18 (11.1)	4/27 (14.8)	4/24 (16.6)	4/18 (22.2)	3/13 (23.1)	11/38 (36.8)
<i>Strongyloides papillosus</i>	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	2/18 (11.1)	8/27 (29.6)	5/24 (20.8)	5 ^a /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)

calves (56% were infected) and the prevalence increased with the age to 85% in 10–11 weeks old calves. *Eimeria bareillyi* was highly prevalent in 2–3 (36%) and 6–7 (76%) weeks age groups (Table 3). Estimated intensity of infection was 8.7×10^4 – 1.5×10^7 OPG, being the highest in 2–5 weeks old calves and decreased with the age (Table 4).

Eggs of *Toxocara vitulorum* were found in all age groups with a prevalence ranging from 11% in 2–3 weeks old buffalo calves to 23% in 10–11 weeks old buffalo calves (Table 3). The EPG value decreased with the age from 7×10^5 to 6.4×10^4 (Table 4), and the presence of adult buffaloes in the same barn with buffalo calves was identified as a risk factor for infection with *T. vitulorum* (Table 5).

Eggs of *Strongyloides papillosus* were detected since 4–5 weeks of age and the prevalence increased with the

age from 4 to 23% (Table 3), with an estimated EPG of 444 on average.

Oocysts of *Cryptosporidium* spp. were detected in the faeces of four calves (3–4 weeks old). DNA of three isolates was successfully amplified and sequenced; two isolates were identified, according to the BLAST search, as *C. ryanae* (GenBank database Acc. nos. MW289064 and MW289065) and clustered with various *C. ryanae* isolates from dairy calves worldwide, particularly those from Asia (China and India). The third isolate (MW289066) grouped in the clade with *C. ryanae*, *C. bovis*, and *C. xiaoi* (Fayer et Santin, 2009), but as a separate sequence (Fig. 3).

Giardia duodenalis was molecularly identified in the faeces of a 9-week old buffalo calf. The revealed nucleotide sequence was identical to that of the isolate AB692776

Table 4. Values of OPG/EPG (mean ± standard error) values for gastrointestinal parasites found in buffalo calves of different age groups

	2–3 weeks	4–5 weeks	6–7 weeks	8–9 weeks	10–11 weeks	Average
<i>Eimeria</i> 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
<i>Toxocara vitulorum</i>	70,415 ± 21,503	26,678 ± 6,420	23,192 ± 13,348	9,186 ± 3,590	6,359 ± 2,934	26,510 ± 7,085
<i>Strongyloides papillosus</i>	-	0	0	0	444 ± 82	444 ± 82

Table 5. Risk factors for infection with *Toxocara vitulorum* in buffalo calves

	Frequency (prevalence)	Odds Ratio	95% Confidence interval Odds Ratio	P
Young buffaloes				
1–2 animals (n = 24)	2 (12.5)	Ref.		
3–4 animals (n = 14)	9 (91.7)	19.80	3.22–121.47	0.001
Adult buffaloes				
1–2 animals (n = 22)	2 (13.6)	Ref.		
3–5 animals (n = 16)	9 (68.8)	12.86	2.22–74.54	0.004
Cattle				
Negative (n = 29)	10 (44.8)	Ref.		
Positive (n = 9)	1 (11.1)	0.24	0.03–2.18	0.20

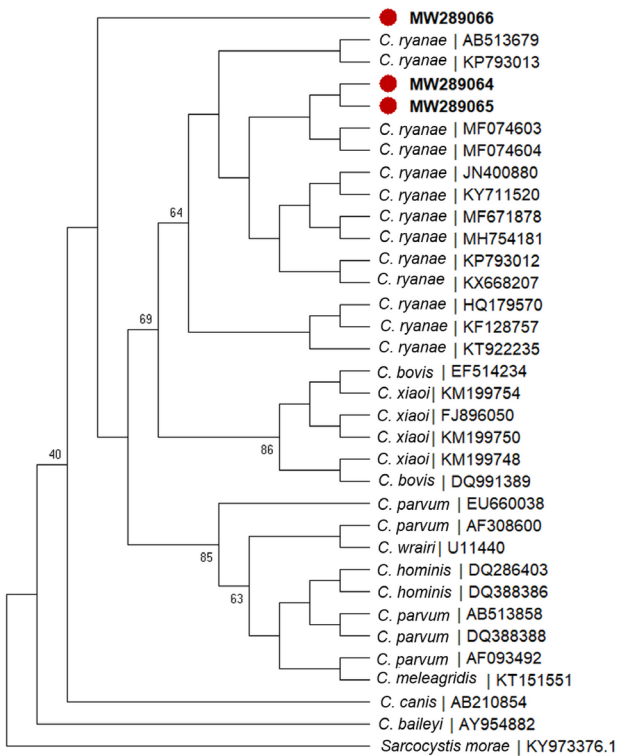


Fig. 3. Maximum Likelihood analysis. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Outgroup taxon analysis.

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 2–11 weeks old from northwestern Romania. Three genera of parasitic protists (species of *Eimeria* and *Cryptosporidium*, and *Giardia duodenalis*) and two nematodes (*Toxocara vitulorum* and *Strongyloides papillosus*) were found. These GIT parasites

are the most common found in buffalo calves worldwide (Ribeiro et al. 2000, Naag et al. 2015).

In the present study, coccidia of the genus *Eimeria* were the most prevalent GIT parasites and they were detected in 84% of buffalo calves. This ubiquitous parasitic protist has a worldwide distribution in water buffaloes which could be infected with at least 12 species of *Eimeria*, 11 of them being of cattle origin (El-Alfy et al. 2019). So far, *Eimeria bareillyi* is the only buffalo-specific species, non-transmissible to cattle, and is highly pathogenic to young calves (Dubey 2018). 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.

Infections with *E. bareillyi* have been reported worldwide (Dubey 2018) including three reports from Europe, i.e., in Italy (Fusco et al. 1997, Guarino 1997) and the Netherlands (Dubey et al. 2008). The prepatent period is 12–15 days after experimental infection with 350,000–15,000,000 oocysts of *E. bareillyi* (see Dubey 2018). In natural infections, oocysts of *E. bareillyi* were detected at 13 days of age (Bastianetto et al. 2008), whereas oocysts of other species, e.g., *Eimeria bovis*, *E. ellipsoidalis*, *E. auburnensis* and *E. zuernii*, were noticed as early as 2–7 days of age (Bastianetto et al. 2007). Therefore, a study on buffalo calves from *E. bareillyi*-infected farm and involving daily examination of faecal samples from the first day of birth is required to detect whether oocysts of *E. bareillyi* are excreted in the first week of life.

The second most prevalent parasite in the present study was *Taxocara vitulorum* (37%), which is frequently found in tropical and sub-tropical regions (Biswas et al. 2014, Alam et al. 2016), and less often in temperate climates such as Europe. This parasite can cause significant morbidity and mortalities in calves (Roberts et al. 1990, Prasad et al. 2010). We noticed the eggs of *T. vitulorum*, but not the mature worms, in the faeces of the examined calves of all age groups. Larvae of *T. vitulorum* are passed in great numbers in the colostrum of buffalo cows 2–5 days post-calving. Nematodes mature in the intestine of the calves within 10 days and eggs are passed in faeces approximately at the

third week of life. Adult worms are then expelled from the intestine by the fifth month of age (Roberts 1990, Roberts et al. 1990). In Europe, this nematode parasite has been reported at farms of cattle (Scala et al. 2001, Diakou and Papadopoulos 2002, Jones et al. 2009, Borgsteede et al. 2012, Chihai et al. 2012, Venjakob et al. 2017) and bisons (Goossens et al. 2007). In western Romania, the parasite was detected in 0.3% of 303 faecal samples from cattle (Sirbu et al. 2020).

Additionally, eggs of *S. papillosus* were found in 16% of the examined buffalo-calves in the present study, and the prevalence increased with the age. A lower prevalence (3%) was detected in water buffaloes (adults, heifer/steers and calves) from Italy (Rinaldi et al. 2009). However, higher values have been reported worldwide: 29% in India (Jyoti et al. 2014); 59% in Mexico (Ojeda-Robertos et al. 2017), and 87% in 3 months old calves in Sri Lanka (Roberts and Fernando 1990, Jyoti et al. 2014, Ojeda-Robertos et al. 2017).

Although species of *Strongyloides* Grassi, 1879 infect all age groups, clinical signs such as diarrhea and malnutrition are frequently seen only in young animals (Thamsborg et al. 2017). Sudden deaths were reported in naturally and experimentally infected calves and EPG values in dead calves varied from 52,000 to 411,000 (Taira and Ura 1991). *Strongyloides*-infected calves from our study presented low EPG values (under 1,000). Considering the high pathogenicity of the parasite, calves with high EPG (> 10,000) should be treated (Thamsborg et al. 2017).

Our study demonstrated infection with cryptosporidia in 4 (11%) buffalo calves; this prevalence is comparable to earlier studies worldwide (Helmy et al. 2013, Ma et al. 2015, Ibrahim et al. 2016). However, our prevalence is much lower than that from buffalo calves (2–4 months) from Egypt (40%) and Brazil (54%) (Aquino et al. 2015, Aboelsoued et al. 2020). Compared to cattle (25–29%) from the country of study (Romania), the obtained prevalence in buffalo calves is lower (Bejan et al. 2008, Imre et al. 2011). DNA from three of the four *Cryptosporidium* isolates was successfully amplified and sequenced, and the obtained sequences revealed similarity with *C. ryanae* in two samples, whereas the third isolate was unidentified but

related genetically to the bovine *C. ryanae* and *C. bovis*, as well as the ovine *C. xiaoi*. *Cryptosporidium ryanae* is the most common species of *Cryptosporidium* reported worldwide in buffaloes (Martins et al. 2018, Russell et al. 2020). In Romania, *Cryptosporidium parvum* was reported in cattle and as the predominant species in lambs (Imre et al. 2011, 2013, Vieira et al. 2015). 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.

A single sample was positive for infection with *G. duodenalis* and sequence analysis revealed it was the assemblage E. This assemblage is non-zoonotic and commonly circulates among different farm animals, including cattle, sheep, goat and horses (Heyworth 2016), and has been detected together with the assemblage A in buffaloes from Italy (Caccio et al. 2007), Australia (Caccio et al. 2007, Abeywardena et al. 2013, Helmy et al. 2013), and Egypt (Helmy et al. 2013).

Generally, buffaloes are considered robust animals 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 species of *Eimeria*, and to the infection with *T. vitulorum*. Moreover, the infection risk for *T. vitulorum* increased when more than two buffaloes are kept in the same barn. Multiple infections and simultaneous occurrence in the same region of the intestine can adversely affect the growth of buffaloes. These data pointed out the need for early coccidiostatic and antihelminthic treatments in buffalo calves, but they do not seem to be a source of zoonotic *C. parvum* and *G. duodenalis* in our area.

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