

Intestinal coccidia (Eucoccidia, Sporozoa, Apicomplexa) of some amphibia in Azerbaijan

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Summary

Cryptosporidium (Coccidia, Apicomplexa) oocysts were detected by carbol-fuchsin staining in feces of amphibia captured in different regions of Azerbaijan. Oocysts were released by 20 specimens of 39 examined *Bufo viridis* (prevalence 51.2%), 3 of 3 examined *Bufo verrucosissimus* (100%), 9 of 28 examined *Rana ridibunda* (32.1%). Invasion intensiveness varied from 12 to 30 oocysts per 1000 fields of vision. *Cryptosporidium* oocysts found in various amphibian species differed from each other by size and shape.

Key words: Amphibia, oocysts, *Cryptosporidium*, light microscopy

Introduction

Coccidia, the intracellular parasitic protozoa, are widespread in invertebrate and vertebrate animals. In Azerbaijan coccidia were found to parasitize over 60 species of mammals, birds, and reptiles (Gaibova, 2004). Circulation of some coccidia between animals and the environment, their life cycles, and host-parasite relations at the organism and cellular levels has been studied. However, very little is known about amphibian coccidia. Two species of *Eimeria* were found in amphibian dwelling in the Greater Caucasus (Musaev and Gaibova, 1995). Two years before we managed to find cryptosporidian oocysts in several specimens of toads and frogs (Mamadova, 2008). In the present paper new data on the prevalence of cryptosporidia in some amphibian species from various regions of Azerbaijan are presented.

Material and methods

Fecal samples were collected from 39 European green toads (*Bufo viridis*), 40 marsh frogs (*Rana ridibunda*), 1 Caucasian Toad (*Bufo verrucosissimus*) and 2 European tree frogs (*Hyla arborea*). The European green toad and marsh frog are widespread in Azerbaijan. The marsh frog can be found in permanent basins throughout the country, in mountains, forests, plains, and in semi-desert landscapes. The European green toad is found both in temporary (puddles, drying runnels) and permanent but shallow basins only during periods of reproduction. The Caucasian toad and European tree frog are rare species in Azerbaijan (Ganiyev and Nuriyev, 2000).

All amphibia were captured in spring, summer and autumn (April–October) in two areas of Azerbaijan: in the Lankaran lowland at the Caspian



Fig. 1. Map of the study area. Arbitrary symbols: □ – *Bufo viridis*, △ – *Bufo verrucosissimus*, □ – *Hyla arborea*, ○ – *Rana ridibunda*.

seaside near the town of Astara and in the Absheron peninsula of the Greater Caucasus in the Gobustan, Samur-Devechi and Gabala districts. Most species were found among amphibians captured in the Lenkeran area: 4 toads (3 European green toads and 1 Caucasian toad), 2 European tree frogs and 19 marsh frogs in the Lankaran lowland, and 9 marsh frogs in the vicinity of Astara. In the Absheron peninsula 36 European green toads were captured; 9 marsh frogs were captured in the Samur-Devechi channel, 1 in Gobustan and 2 in the vicinity of Gabala (Fig. 1).

The captured animals were held for 7-10 days in the aquariums specially equipped for frogs in the Laboratory of Protistology, Institute of Zoology, National Academy of Azerbaijan. The frogs had enough food but they practically did not eat in the laboratory conditions. Feces were collected once from each animal. To detect *Cryptosporidium* oocysts feces were smeared on slides. The slides were stained with carbol-fuchsin and methylene green after Ziehl-Nilsen in modification of Henriksen and Pohlenz (Henriksen and Pohlenz 1981). The smears were dried thoroughly in the air, then fixed with 95 % methanol for 5 minutes and dried again. Then the preparations were held for a second above the flame for 3-5 times. The slides were placed into the carbol-fuchsin solution for 20-40 minutes then rinsed with distilled water and additionally stained with methylene green. By this technique

the cryptosporidian oocysts got stained pink and the concomitant microflora – green. Additionally, fecal samples of every specimen examined were centrifuged in a solution of supersaturated sodium chloride. All the oocysts found were measured and length to width shape indices were calculated. Oocyst sizes were compared. Statistical significance of differences among oocysts was estimated by Student's criterion and expressed as mean \pm SE. Invasion intensiveness was measured as a number of oocysts in fecal smears per 1000 fields of vision at the magnification 1000 \times . Percentage of animals that released oocysts, indicated the prevalence of infection.

Results

Cryptosporidian oocysts were found in feces of all examined amphibian species except for European tree frogs (Table 1). Oocysts of other coccidian species were not found.

Cryptosporidia were found in European green toads in the 'Absheron' and 'Lankaran' populations. Prevalence of cryptosporidia in European green toads was 47.2%. All toads captured in the Lankaran lowland (3 European green toads and 1 Caucasian toad) were infected. In the marsh frogs captured in the Lankaran lowland and in vicinity of Astara the prevalence values were 36.8 and 22.2%, respectively.

Table 1. Prevalence and oocyst size of *Cryptosporidium* spp. found in Azerbaijan amphibia.

Host species	Region of sampling	Fraction of infected animals* (%)	Intensiveness of infection (mean)	Size	
				length μm , mean \pm SE (n**)	width l/w
<i>Bufo viridis</i>	Absheron	17/36 (47.2)	12	5.71 \pm 0.02 \times 5.39 \pm 0.01, (n=24)	
<i>B. viridis</i>	Lankaran	3/3	12	4.41 \pm 0.04 \times 4.14 \pm 0.04, (n = 16)	
Total:		20/39 (51.2)			
<i>B. verrucosissimus</i>	Lankaran	1/1	12	6.31 \pm 0.01 \times 5.01 \pm 0.01, (n = 25)	
<i>Rana ridibunda</i>	Lankaran	7/19 (36.8)	16	5.01 \pm 0.02 \times 4.76 \pm 0.07, (n = 27)	
<i>R. ridibunda</i>	Astara	2/9 (22.2)	30	4.44 \pm 0.01 \times 4.38 \pm 0.02, (n = 25)	
Total:		9/28 (32.1)			

Notes: * number of the infected amphibians per number of the examined ones

** number of measured oocysts

The intensiveness of invasion (II) of European green toads captured in the Lankaran region with cryptosporidian oocysts averaged 12 (from 8 to 22 oocysts). The II of the single Caucasian toad found in the Lankaran was the same as in European green toads (12 oocysts per 1000 fields of vision).

The II of the marsh frogs captured in the Lankaran region with cryptosporidian oocysts averaged 16 (from 6 to 36), and the average of those captured in the Astara region was 30. In one marsh frog of the Astara population the II was 140 oocysts per 1000 fields of vision. That specimen was not taken into account when calculating the mean II for oocysts from Astara marsh frogs.

Average sizes of the oocysts of European green toads found in Absheron peninsula were 5.71 \pm 0.02 \times 5.39 \pm 0.01 μm , length-to-width ratio (l/w) = 1.06 \pm 0.003, and in the Lankaran region - 4.41 \pm 0.04 \times 4.14 \pm 0.04 μm , l/w = 1.1 \pm 0.01 (Fig. 2, A and B).

The oocysts found in the Caucasian toads were distinctly different from those found in the European green toads both of the 'Absheron' and the 'Lankaran' population. Their average sizes were 6.31 \pm 0.01 \times 5.01 \pm 0.01 μm . Generally all the oocysts had an elongate oval shape (l/w = 1.24) (Fig. 2, C) but sometimes round oocysts, about 5.01 μm in diameter, could be found, l/w = 1.0.

The oocysts found in the marsh frogs captured in the Lankaran and Astara regions were not different in shape from oocysts of the European green toads from the Lankaran region. Most oocysts were round, some were slightly elongate. The sizes of the oocysts from the marsh frogs captured in Lankaran were 5.01 \pm 0.02 \times 4.76 \pm 0.07 μm , l/w = 1.05 \pm 0.003 μm , and in the marsh frogs from Astara they were 4.44 \pm 0.01 \times 4.38 \pm 0.02 μm , l/w = 1.05 \pm 0.01 (Fig. 2 D and E).

Discussion

Cryptosporidia were reported in amphibia for the first time in 1986. They were found in frogs in a zoo (Fayer, 1997). Cryptosporidia were then detected in laboratory frogs that died of unknown intestinal disease (Green et al., 2003). Some features of penetration of *Cryptosporidium* sp. into toad (*Bufo* sp.) intestinal cells were reported (Vigulova et al, 2007). *Cryptosporidium fragile* was reported from black-spined toads (*Duttaphrynus melanostictus*) imported from Malaysia to Czech Republic (Jirku et al. 2008).

One can see from the results of the present study that cryptosporidia are found in amphibia of Azerbaijan rather frequently. About a half (47.2%) of the European green toads captured in the Absheron peninsula released oocysts. There were many specimens infected with cryptosporidia among the marsh frogs of Lankaran region as well (32.1%).

Oocysts of *Cryptosporidium* sp. in 6 European green toads (*Bufo viridis*) and 1 marsh frog (*Rana ridibunda*) were previously reported from Azerbaijan (Mamadova, 2008). The II of the European green toad with cryptosporidia differed in different years in the Absheron peninsula. In toads captured in 2007 the II averaged 36 oocysts (from 12 to 55). The II in 2008 was significantly lower averaging only 12 oocysts.

The oocysts found in 3 amphibian species significantly differed by morphometric features which enabled us to classify all the oocysts into 4 categories. The oocysts found in the Caucasian toad were the largest; they differed from the oocysts found in the two other amphibian species by their elongate oval shape. In each of the amphibian species, the European green toad and the marsh frog, we found two different kinds of oocysts. The oocysts released from the Lankaran and Absheron European green toads were different and the difference was

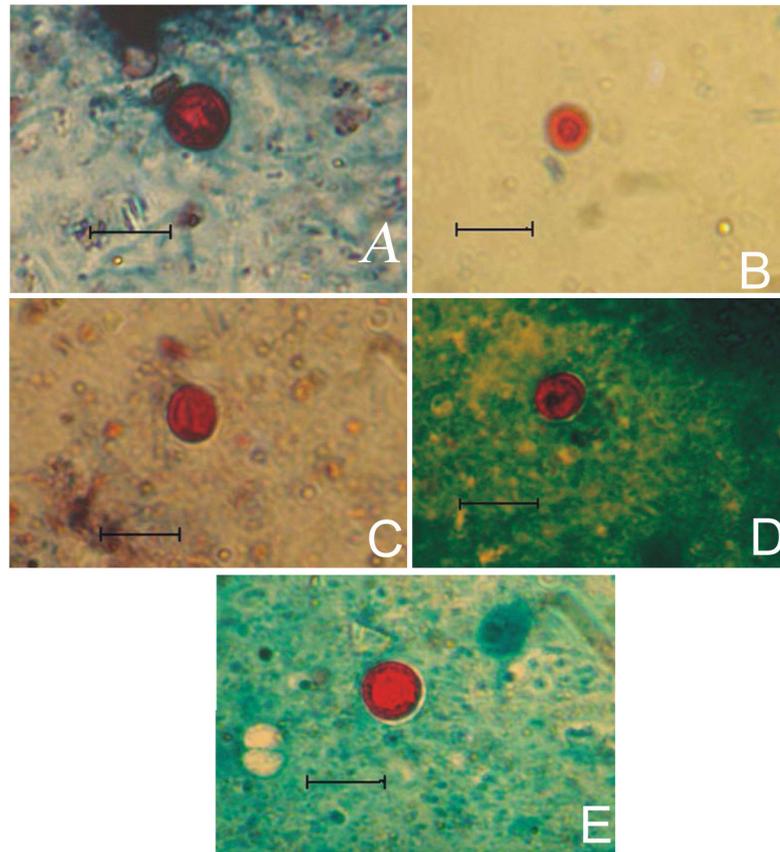


Fig. 2. *Cryptosporidium* oocysts found in isolates in Amphibia staining in carbol-fuchsin. A – An oocyst in isolate of *B. viridis* (Absheron population), B – the same for Lenkaran population, C – the same for *B. verrucosissimus*, D – the same for *R. ridibunda* (Lenkaran population), and E – the same for *R. ridibunda*, captured in the vicinity of Astar town. Scale bars: 10 μ m.

statistically significant. The situation was the same for the Lenkaran and Astar frogs. However, measurements of oocysts from Caucasian toads of Lenkaran were not significantly different from those released by marsh frogs of Astar, i.e. the difference was not statistically significant.

The sizes of cysts from European green toads captured in Absheron peninsula before (Mamadova, 2008) were not different from those described in this study.

It is known that 19 of the 20 valid *Cryptosporidium* species of mammals, birds, reptiles and fishes are not significantly distinguishable in terms of morphology. The sizes of most species do not surpass 4–6 μ m. Yet some species differ by morphometric parameters. The biggest oocysts (8.25 - 6.3 μ m, l/w = 1.3) were found in *C. galli* from birds and the smallest ones - in *C. molnari* (4.72 - 4.47 μ m, l/w = 1.05) from fish (Xiao et al., 2004). The sizes of the *Cryptosporidium* oocysts from *Bufo verrucosissimus* are similar to

oocysts of *C. serpentis* from snakes and lizards (Xiao et al., 2004). The oocysts released by *Rana ridibunda* from the Lenkaran region were close in size to *C. saurophilum* from snakes and lizards (Xiao et al., 2004). The sizes of the oocysts from *Bufo viridis* of the Lenkaran population and *Rana ridibunda* of the Astar population are close to *C. molnari* from fishes (Xiao et al., 2004). Morphometric features of the *Cryptosporidium* oocysts from *Bufo verrucosissimus* are close to those of the only one valid amphibian cryptosporidian, *C. fragile* (Jirku M. et al., 2008). Most researchers state that morphological features of cryptosporidian oocysts are not sufficient species criteria for *Cryptosporidium*. Therefore we refrain from species definition for cryptosporidian oocysts reported in amphibia herein, until life cycle studies and DNA analyses are performed. We intend to conduct cross infection with the *Cryptosporidium* oocysts found by us, and to study life cycles, and then to conduct DNA analysis.

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