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ORIGINAL ARTICLE

Toxoplasma gondii, Neospora caninum and *Encephalitozoon cuniculi* in Animals from Captivity (Zoo and Circus Animals)

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ABSTRACT

Problems with parasitic infections are common in zoological gardens and circuses. In some animals it can lead to several disorders such as systemic disease, reproductive disorders (abortions and neonatal mortality), and even to death if severe illness is untreated. Thus, the aim of this study was to evaluate the prevalence of three common parasites in 74 animals from three zoos, and four circuses in Southern Italy. Antibodies to *Toxoplasma gondii, Neospora caninum,* and *Encephalitozoon cuniculi* were detected in 51%, 12%, and 20% of animals, respectively. Co-infections of *T. gondii* and *N. caninum* were reported in seven animals (9%) and co-infection of *T. gondii* and *E. cuniculi* in one animal. *T. gondii*, *N. caninum* and *E. cuniculi* seroprevalence differed in type of diet ($P \leq 0.0001$; $P \leq 0.037$ and $P \leq 0.004$, respectively). *T. gondii* and *E. cuniculi* seroprevalence also differed in animal families ($P \leq 0.0001$) and according to type of housing ($P \leq 0.003$), respectively. Statistical differences were not found in other characteristics (gender, age, country of birth, origin, and contact with cats or dogs). This is the first serological study focusing on protozoan and microsporidian parasites in zoo and circus animals from Southern Italy and the first detection of antibodies to *E. cuniculi* in camels in Europe.

CAPTIVE animals are generally more often under high stress situations than wildlife. They are also at higher risk of parasitic infection because of their breeding in closed yards with possibly higher concentrations of oocysts or spores in environment. Coccidia, *Toxoplasma gondii*, and *Neospora caninum* have an indirect life cycle with a wide range of warm-blooded animals as intermediate hosts, and felids and canids as definitive hosts, respectively. Deaths caused by toxoplasmosis were recorded in various animal species bred in captivity (Pallas cats, New world monkeys, canaries, lemurs, finches, Australasia marsupials etc.). Furthermore, not only zoo animals but also humans (especially keepers and veterinarians) could be at risk of exposure to *T. gondii* infection, especially through oocysts excreted by felids and thus contaminating the zoo area (De Camps et al. 2008; Sedlák and Bártová 2006). Lethal neosporosis was recorded e.g. in aborted fetus of white rhinoceros (*Ceratotherium simum simum*) (Sangster et al. 2010), and in spotted deer (*Axis axis*) (Basso et al. 2014). Microsporidium *Encephalitozoon cuniculi* has a direct life cycle, and is also able to infect a wide range of warm-blooded animals. In captive animals, *E. cuniculi* infection is usually asymptomatic, nevertheless fatal encephalitozoonosis have also been recorded e.g. in tamarins (*Saguinus imperator*) (Juan-Sallés et al. 2006). Infected animals can excrete resistant spores in their urine, faeces, and sputum (Hinney et al. 2016), and thus contaminate their environment that could be the source of infection for other animals and humans, as visitors of zoos and circuses. The occurrence of *T. gondii, N. caninum,* and

E. cuniculi antibodies in animals is an important indicator of circulation of these infections in zoos and circuses. The aim of this study was to determine the seroprevalence of *T. gondii, N. caninum*, and *E. cuniculi* in zoo and circus animals from Southern Italy.

MATERIALS AND METHODS

Animals used

In 2014, blood samples were collected from 74 animals of 14 species belonging to eight families, and coming from captivity (three zoos and four circuses) in Southern Italy. The animals were divided into categories according to the following characteristic: family, gender, age, country of birth, origin, type of housing, and contact with cats or dogs (Table 1).

Sample processing

Serum samples were obtained by centrifugation at 358 *g* for 10 min and stored at −20 °C. All the procedures performed in this study were conducted during the routine health check according to the European Communities Council Directive of 24 November 1986 (86/609/EEC).

IFAT/ELISA assay

Antibodies to *T. gondii* and *N. caninum* were detected by indirect fluorescence antibody test (IFAT, VMRD, Pullman, Chicago, IL) using commercially available *T. gondii* antigen (whole parasite *T. gondii*) and *N. caninum* antigen (whole parasite *N. caninum*) fixed on 12 well slides. Specific conjugates were used for each animal family: anti-bovine IgG (Sigma Aldrich, St. Louis, MO) for watusi cattle and yak, anti-sheep IgG (Sigma Aldrich) for barbary sheep, anti-llama IgG (VMRD) for Camelidae, anti-deer IgG (KPL Inc. Gaithersburg, MD) for Cervidae, anti-horse IgG (VMRD) for Equidae, anti-cat IgG (Sigma Aldrich) for Felidae, and anti-human IgG (Sevapharma, Prague, Czech Republic) for Hominidae. Positive and negative *T. gondii* and *N. caninum* sera (Sedlák and Bártová 2006) were included in both tests. Antibodies to *E. cuniculi* were tested by IFAT using the commercial set MegaScreen Fluoencephalitozoon c. (Megacor Diagnostic, Hörbranz, Austria) with antigen (whole spores of *E. cuniculi*), anti-rabbit FITC conjugate and positive and negative controls (rabbit serum). Sera were diluted with phosphate buffered saline two-fold starting with dilution 1:50; the samples with titres ≥ 50 were considered positive. The glass slides were examined with fluorescence microscope (OLYMPUS BX 41) at 1,000X magnification with oil immersion. In elephants and kangaroo, there are no specific conjugates for IFAT. To overcome this drawback, in this group of animals, the antibodies to *T. gondii* and *N. caninum* were detected by enzyme-linked immunosorbent assay (ID Screen Toxoplasmosis Indirect Multi-species, IDvet, France and ID Screen *Neospora caninum* Indirect Multi-species, respectively) using specific antigens (*T. gondii* antigen P30 and extract of *N. caninum*) and multi species conjugates (protein A and P). Sera were diluted 1:10 and optical densities (OD) were measured spectrophotometrically at 450 nm. For each sample, the ratio of optical densities of examined serum to mean OD of positive control was calculated as S/P (%), according to the formula: S/P (%) = (OD sample/OD positive control) × 100. Samples with the S/P (%) $\geq 50\%$ were classified as positive. Antibodies to *E. cuniculi* were not tested in this group of animals.

Statistical analyses

The data analysis was performed with Pearson chi-square test for independence using STATISTICA Cz 12 (StatSoft, Inc. 2013) or with method Monte Carlo using IBM SPSS Statistics 20. We tested the null hypothesis that seroprevalence of parasites does not differ in family, gender, age, country of birth, origin, type of housing, type of diet, and contact with cats or dogs. The differences were considered statistically significant when *P*-value was ≤ 0.05. In case of rejection of the null hypothesis, Scheffe's multiple comparison method was used. The power of the statistical test was calculated if it was possible.

RESULTS AND DISCUSSION

In the present study, antibodies directed to *T. gondii, N. caninum,* and *E. cuniculi* were detected by IFAT in 51%, 12%, and 20% of animals, respectively (Table 1). Results of prevalence according to animal species are summarized in Table 2. Co-infection of *T. gondii* and *N. caninum* was recorded in seven animals (9%), four lions (*Panthera leo*), two tigers (*Panthera tigris*), and one reindeer (*Rangifer tarandus*). Co-infection of *T. gondii* and *E. cuniculi* was recorded only in one alpaca (*Vicunia pacos*); co-infection of *N. caninum* and *E. cuniculi* was not recorded. Statistical significant difference was found in *T. gondii* prevalence depending on animal families ($P \leq 0.0001$) and type of diet ($P \leq 0.0001$). Scheffe's method showed a difference between Felidae and Bovidae ($P = 0.004$, power = 0.995), Felidae and Camelidae ($P = 0.001$, power = 0.999), Felidae and Equidae ($P = 0.00002$, power = 1), between Equidae and Elephantidae ($P = 0.0088$, power cannot be calculated), and between Carnivores and Herbivores ($P \leq 0.0001$, power = 1). *N. caninum* prevalence statistically differed depending on the type of diet ($P = 0.0365$); Scheffe's method showed a difference between Carnivores and Herbivores ($P = 0.043$, power = 0.532). *E. cuniculi* prevalence statistically differed depending on different way of housing ($P \leq 0.0028$) and type of diet ($P = 0.004$). Scheffe's method showed a difference between couples and groups ($P = 0.0119$, power = 0.748) and Carnivores and Herbivores ($P = 0.007$, power = 0.869). Statistical differences were not found in other characteristics. The results obtained in this study highlight the need for a practical scheme to reduce the presence of these parasites in zoo and circus environments.

Table 1. The results of serological examination (*Toxoplasma gondii, Neospora caninum,* and *Encephalitozoon cuniculi*) according to different characteristic of zoo and circus animals from Italy

Characteristic	Positive/*n* (%)			*P*-value
	T. gondii	*N. caninum*	*E. cuniculi*	
Family				
Bovidae	2/10 (20%)	1/10 (10%)	0/10 (0%)	< 0.0001, 0.126, NT
Camelidae	7/23 (30%)	0/23 (0%)	14/23 (61%)	
Cervidae	2/2	1/2	0/2	
Elephantidae[a]	4/4	0/4	NT	
Equidae	0/8 (0%)	1/8 (13%)	0/8	
Felidae	21/22 (95%)	6/22 (27%)	0/22	
Hominidae	2/4	0/4	0/4	
Macropodidae[a]	0/1	0/1	NT	
Gender				
Male	22/44 (50%)	6/44 (14%)	10/43 (23%)	0.816, 0.731, 0.431
Female	16/30 (53%)	3/30 (10%)	4/26 (15%)	
Age (yr)				
0–1	2/5 (40%)	2/5 (40%)	1/4	0.681, 0.323, 0.546
≥ 2–5	14/31 (45%)	2/31 (6%)	8/31 (26%)	
≥ 6–9	10/19 (53%)	2/19 (11%)	4/19 (21%)	
≥ 10–14	4/5 (80%)	1/5 (20%)	1/5 (20%)	
≥ 15	8/14 (57%)	2/14 (14%)	0/10 (0%)	
Country of birth				
France	1/2	0/2	0/2	0.381, 0.418, 0.083
Holand	1/2	0/2	2/2	
Hungary	1/1	0/1	0/1	
Italy	15/29 (52%)	5/29 (17%)	7/28 (25%)	
Romania	2/4 (50%)	0/4	0/4	
Spain	12/19 (63%)	4/19 (21%)	2/19 (11%)	
Not known	6/17 (35%)	0/17 (0%)	3/13 (23%)	
Origin				
Zoo	11/29 (38%)	2/29 (7%)	7/26 (27%)	0.064, 0.266, 0.287
Circus	27/45 (60%)	7/45 (16%)	7/43 (16%)	
Housing				
Alone	2/4	0/4	1/2	0.822, 0.131, 0.003
Couple	2/6 (33%)	0/6 (0%)	4/5 (80%)	
Group	21/38 (55%)	8/38 (21%)	4/37 (11%)	
Not known	13/26 (50%)	1/26 (4%)	5/25 (20%)	
Diet				
Carnivores	21/22 (96%)	6/22 (27%)	0/22	< 0.0001, 0.037, 0.004
Herbivores	15/48 (31%)	3/48 (6%)	14/43 (33%)	
Omnivores	2/4	0/4	0/4	
Contact with cats				
Yes	27/57 (47%)	6/57 (11%)	11/54 (20%)	0.209, 0.421, > 0.999
No	11/17 (65%)	3/17 (18%)	3/15 (20%)	
Contact with dogs				
Yes	23/41 (56%)	3/41 (7%)	8/38 (21%)	0.363, 0.178, 0.862
No	15/33 (45%)	6/33 (18%)	6/31 (19%)	
Total	38/74 (51%)	9/74 (12%)	14/69 (20%)	

NT = not tested; OD = optic density.

Antibodies to *T. gondii, N. caninum,* and *E. cuniculi* were tested by indirect fluorescence antibody test (IFAT).

[a]In Elephantidae and Macropodidae, antibodies to *T. gondii* were detected by enzyme-linked immunosorbent assay (ELISA); S/P (%) was calculated according to the formula: S/P = (OD sample/OD positive control) × 100. Samples with the S/P (%) ≥ 50% were positive. Antibodies to *N. caninum* were detected by complement enzyme-linked immunosorbent assay (cELISA); % I (% inhibition) was calculated according the formula: % I = 100 − (OD sample × 100/mean OD negative control). Samples with 30% inhibitions were considered to be positive.

In feline carnivores, antibodies to *T. gondii* and *N. caninum* were detected in 95% and 27% of 22 animals, respectively with co-infection in 27% of animals (in four lions and two tigers). Higher *T. gondii* antibody prevalence compared to *N. caninum* is in accordance with other studies: 63% and 50%, respectively in wild felids living in

Table 2. The results of serological examination (*Toxoplasma gondii*, *Neospora caninum,* and *Encephalitozoon cuniculi*) according to species of zoo and circus animals from Italy

		T. gondii		*N. caninum*		*E. cuniculi*	
Family	Species	Positive/*n* (%)	Range of titers	Positive/*n* (%)	Range of titers	Positive/*n* (%)	Range of titers
Bovidae	Barbary sheep (*Ammotragus lervia*)	1/4	200	0/4	–	0/4	
	Domestic Yak (*Bos mutus f. grunniens*)	0/3	–	0/3	–	0/3	
	Watussi (*Bos primigenius f. taurus*)	1/3	100	1/3	50	0/1	
Camelidae	Alpaca (Lama guanicoe *f. pacos*)	2/4	50	0/4	–	3/4	50
	Bactrian camel (*Camelus bactrianus*)	2/9 (22%)	50	0/9 (0%)	–	6/9 (67%)	50–1,600
	Dromedary camel (*Camelus dromedarius*)	2/5 (40%)	50	0/5 (0%)	–	2/5 (40%)	50
	Llama (*Lama guanicoe f. glama*)	1/5 (20%)	50	0/5 (0%)	–	3/5 (60%)	50
Cervidae	Reindeer (*Rangifer tarandus*)	2/2	100, 800	1/2	50	0/2	
Elephantidae[a]	Indian elephant (*Elephas maximus*)	4/4	130–190	0/4	–	NT	
Equidae	Grant's zebra (*Equus quagga boehmi*)	0/8 (0%)	–	1/8 (13%)	50	0/8 (0%)	
Felidae	Bengal tiger (*Panthera tigris bengalensis*)	8/8 (100%)	200–1,600	2/8 (25%)	50–1,600	0/8 (0%)	
	Lion (*Panthera leo*)	13/14 (93%)	100–3,200	4/14 (29%)	50–800	0/14 (0%)	
Hominidae	Common chimpanzee (*Pan troglodytes*)	2/4 (50%)	50, 400	0/4	–	0/4	
Macropodidae[a]	Bennett's wallaby (*Macropus rufogriseusfrutica*)	0/1	–	0/1	–	NT	
Total	14 species	38/74 (51%)		9/74 (12%)		14/69 (20%)	

NT = not tested; OD = optic density.

Antibodies to *T. gondii, N. caninum,* and *E. cuniculi* were tested by indirect fluorescence antibody test (IFAT).

[a]In Elephantidae and Macropodidae, antibodies to *T. gondii* were detected by enzyme-linked immunosorbent assay (ELISA); S/P (%) was calculated according to the formula: S/P = (OD sample/OD positive control) × 100. Samples with the S/P (%) ≥ 50% were positive. Antibodies to *N. caninum* were detected by complement enzyme-linked immunosorbent assay (cELISA); % I (% inhibition) was calculated according the formula: % I = 100 − (OD sample × 100/mean OD negative control). Samples with 30% inhibitions were considered to be positive. Antibodies to *E. cuniculi* were not tested in this group of animals.

captivity in Brazil zoo (André et al. 2010), 44% and 7% in felids held privately or in zoos in the United States (Spencer et al. 2003), and 93% and 12% in felids from zoo in the Czech Republic (Sedlák and Bártová 2006). In contrast, Kamga-Waladjo et al. (2009) found higher prevalence of *N. caninum* antibodies (100%) compared to *T. gondii* (43%) in seven lions from Senegal's zoo. The main source of *T. gondii* and *N. caninum* infection for carnivores is consumption of feed containing *T. gondii* or *N. caninum* tissue cysts (Dubey and Schares 2011; Elmore et al. 2010) or consumption of wildlife such as rodents and birds with free access to zoo. These parasites were detected by PCR in brains of wild rodents from Austria (Fuehrer et al. 2010) and in livers of wild rodents from the Czech Republic (Machačová et al. 2016).

Most of herbivores from our study were bred in groups or couples in open areas with possible contact with wildlife. Relatively high prevalence of *T. gondii* (30%) and *E. cuniculi* (61%) antibodies was found in camelids with co-infection in one 4-year old female alpaca from zoo. According to our knowledge, this is the first detection of antibodies to *E. cuniculi* in camelids. Co-infection of *T. gondii* and *N. caninum* was detected in 3-year old male reindeer from zoo. Co-infection of these two parasites was recorded also in cervids from zoo in the Czech Republic; however, only antibodies to *N. caninum* were detected in deer (Sedlák and Bártová 2006). Antibodies to *T. gondii* were also found in all four tested elephants (two from zoos and two from circuses with age ranging from 32 to 57 yr), while they were negative for *N. caninum* antibodies. Bennett's wallaby (*Macropus rufogriseus*) that was 5 mo old was negative for both *T. gondii* and *N. caninum* antibodies. Herbivores are mostly infected by water or feed contaminated with *T. gondii* or *N. caninum* oocysts, shed by felids and canids, respectively (Dubey 2010). De Camps et al. (2008) tested 34 trapped feral domestic cats from three zoos in the USA, and detected *T. gondii* antibodies in 10 cats (29%), that could be a source of oocysts contaminating the environment of a zoo.

In our study, antibodies to *T. gondii* were detected in two of four common chimpanzees (*Pan troglodytes*); the number of tested animals was very limited for any conclusion. Nevertheless, primates are highly sensitive to *T. gondii* infection (Dubey 2010). Antibodies to *T. gondii* were also recorded e.g. in 73% of Hominidae from zoo in the Czech Republic (Sedlák and Bártová 2006).

The present study is the first survey conducted on protozoa and microsporidia in animals from zoos and circuses in Southern Italy. Considering that zoos and circuses are frequently attended by children, parasitological monitoring should be introduced in the management of these structures with attention to these potential zoonotic microorganisms. Animal handlers should keep animals in good hygienic condition to avoid contact of zoo animals with feral cats, dogs, and rodents. Feed (hay, grain or

vegetable) should be stored in conditions to prevent its contamination with parasitic oocysts or spores. It is also highly recommended to use meat frozen below −13 °C to devitalize *T. gondii* in meat used for carnivores (De Camps et al. 2008).

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