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Exposure to *Neospora* spp. and *Besnoitia* spp. in wildlife from Israel

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ABSTRACT

Neosporosis and besnoitiosis, caused by cyst-forming protozoa *Neospora caninum* and *Besnoitia besnoiti*, respectively, are parasitic infestations of livestock in Israel. These parasites cause significant economic losses in cattle due to reproductive and productive disorders. Both parasites have been detected in several wild ruminant species throughout other regions of the world, while the existence of a sylvatic life cycle in Israel remains uncertain. Thus, a wide panel of 871 sera from two wild carnivores and nine wild ruminant species were tested. All sera were first analysed by MAT for an initial screening and positive samples were confirmed *a posteriori* by Western blot. Additionally, a complementary IFAT was used for the detection of antibodies against *N. caninum*. *Neospora* antibodies were present in six out of the 11 species investigated, whereas *Besnoitia* antibodies were undetected. Golden jackal, red fox, addax, Arabian oryx, Persian fallow deer, mouflon, mountain gazelle, Nubian ibex, scimitar horned oryx and water buffalo were seropositive against *N. caninum* infection by IFAT and/or MAT. Moreover, the presence of *Neospora* spp.-specific antibodies was confirmed by Western blot in golden jackal (6/189; 3.2%), red fox (1/75; 1.3%), Persian fallow deer (13/232; 5.6%), mouflon (1/15; 16.7%), Nubian ibex (22/55; 40%) and water buffalo (12/18; 66.7%). Addax (1/49) and water buffalo (1/18) were MAT-seropositive against *B. besnoiti* but were seronegative by Western blot. Hence, *Neospora* sylvatic cycle is present in Israel and may cross over to a domestic life cycle. In contrast, wildlife species investigated are unlikely to present a risk of transmitting *Besnoitia* to livestock in Israel.

Neosporosis and besnoitiosis are protozoan reproductive and productive diseases of cattle and are caused by *Neospora caninum* and *Besnoitia besnoiti*, respectively (Dubey et al., 2017; Alvarez-García et al., 2013). Both diseases are present in Israel (Fish et al., 2007; EFSA, 2010). Specifically, more than 45% of cows in dairy farms were seropositive to *N. caninum*, and *Neospora*-associated abortions have also been reported (Fish et al., 2007). Bovine besnoitiosis was widely reported in the 1960s; however, the current epidemiological situation is unknown even after several decades of using a live tachyzoite vaccine from an Israeli bovine isolate (unpublished data).

Although domestic cattle act as intermediate host for both parasites, both infections have also been diagnosed in wild animal species. Dogs, dingoes, coyotes and grey wolves are definitive hosts for *N. caninum*, whereas the definitive host for *B. besnoiti* is still unknown despite the fact that both, domestic and wild cats, have been suggested (Alvarez-García et al., 2013; Dubey et al., 2017). Nevertheless, the putative role of a sylvatic life cycle in the epidemiology of both diseases in cattle has not been fully elucidated (Gondim, 2006; Alvarez-García et al., 2013). The location of Israel at the border of four biogeographical regions contributes to the abundance of a wide range of wild animals. However, the search for specific *N. caninum* antibodies has been restricted to wild carnivores and crows (Steinman et al., 2006; Salant et al., 2015).

The detection of specific antibodies in wildlife is a challenge due to: i) the use of non-validated serological tools and the absence of reference sera (Gutiérrez-Expósito et al., 2016; Bartova et al., 2017); ii) low sample quality that leads to the degradation of immunoglobulins; iii) lack of species-specific secondary antibodies and iv) cross-reactions with closely related parasites. Therefore, Donahoe et al. (2015) suggested the use of more than one serological technique to obtain accurate results.

In the present study, we evaluated the presence of specific antibodies against *Neospora* spp. and *Besnoitia* spp. parasites in a wide panel

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Table 1
Detection of specific anti-*Neospora* antibodies by Western blot in IFAT and/or MAT positive sera.

Species		Samples (n)	MAT[a] (%, nº positive/ nº tested)	IFAT[a] (%, nº positive/ nº tested)	Western blot confirmation (%, nº positive/ nº sample tested)[b]		Seroprevalence (%)[c]
					MAT	IFAT	
Carnivores	Golden jackal (*Canis aureus*)	189	**15.3 (29/189)**	**2.1 (4/189)**	**20 (6/29)**	**50 (2/4)**	**3.2 (6/189)**
	Red fox (*Vulpes vulpes*)	75	**12.0 (9/75)**	**1.3 (1/75)**	**11.1 (1/9)**	**100 (1/1)**	**1.3 (1/75)**
Ruminants	Addax (*Addax nasomaculatus*)	49	**24.4 (12/49)**	0.0 (0/49)	0.0 (0/12)	–	0.0 (0/12)
	Arabian oryx (*Oryx leucoryx*)	60	**5.0 (3/60)**	0.0 (0/60)	0.0 8 (0/3)	–	0.0 (0/3)
	Persian fallow deer (*Dama mesopotamica*)	232	**23.3 (54/232)**	**5.17 (12/232)**	**24.1 (13/54)**	**100 (12/12)**	**5.6 (13/232)**
	Mouflon *(Ovis orientalis)*	15	**40 (6/15)**	**6.7 (1/15)**	**16.7 (1/6)**	**100 (1/1)**	**6.7 (1/15)**
	Mountain gazelle (*Gazella g. gazella*)	123	**18.7 (26/123)**	**2.4 (3/123)**	0.0 (0/26)	0.0 (0/3)	0.0 (0/123)
	Nubian ibex (*Capra nubiana*)	55	**65.4 (36/55)**	**27.3 (15/55)**	**61.1 (22/36)**	**100 (15/15)**	**40.0 (22/55)**
	Roe deer (*Capreolus capreolus*)	19	0.0 (0/19)	0.0 (0/19)	–	–	0.0 (0/0)
	Scimitar horned oryx (*Oryx dammah*)	36	**2.7 (1/36)**	0.0 (0/36)	0.0 (0/1)	–	0.0 (0/1)
	Water buffalo (*Bubalus bubalis*)	18	**77.8 (14/18)**	**72.2 (13/18)**	**85.7 (12/14)**	**84.6 (11/13)**	**66.7 (12/18)**
Total		*871*	*21.8 (190/871)*	*5.9 (52/871)*	*18.4 (35/190)*	*85.7 (42/49)*	*6.3 (55/871)*

[a] Antibody titer equal or higher than 1:200.
[b] Only positive result by either IFAT or MAT were tested by WB.
[c] Number of WB-positive animals/total of sampled animals.

of wild animals. Three serological assays were used to detect *Neospora* spp. and, two were used for *Besnoitia* spp.

A total of 871 samples from two wild carnivorous species and nine ruminant species were analysed (Table 1). Between 2006 and 2013, blood samples were collected from animals during regular monitoring and from dead animals at different locations in North, Central and South Israel by the Nature and Parks Authorities of the country (Fig. 1). Halula preservation (area A) and Hai-Bar Nature Reserve (area B) are fenced areas located in the north, whereas areas C (Ein Gedi Reserve), D (Ein Hazeva) and E (Yotvata) are open areas located in central and southern Israel. Age and sex data for sampled animals were unavailable. Most of samples from wild carnivores were collected from dead animals whereas the majority of samples from wild ruminants were from live animals. The serum was obtained by centrifugation and maintained at −80 °C until tested. All sera were initially analysed by the modified agglutination test (MAT) (Packham et al., 1998; Waap et al., 2011). In addition, a complementary immunofluorescence antibody test (IFAT) was used for the detection of specific *N. caninum* antibodies (Fish et al., 2007). Subsequently, samples with MAT and/or IFAT titres ≥1:200 were posteriorly confirmed using *N. caninum* and *B. besnoiti* tachyzoite-based Western blot as a gold standard technique (Alvarez-García et al., 2002; García-Lunar et al., 2013). An animal was considered positive if the presence of specific antibodies was confirmed by Western blot analysis.

Tachyzoites of the Nc1 *N. caninum* isolate (Dubey et al., 1988) and BbSp-1 isolate of *B. besnoiti* (Fernández-García et al., 2009) were grown *in vitro* as previously described (Fernández-García et al., 2009). Later, these tachyzoites were pelleted and frozen at −80 °C for Western blot tests or resuspended in PBS and formalin-fixed for IFAT and MAT.

Neospora spp.-based MAT was carried out as previously described (Packham et al., 1998). Sera were diluted serially two-fold from 1:100 to a final dilution of 1:12,800. Due to the lack of a reference panel of sera and to avoid overestimating positive results, a conservative cut off of 1:200 was selected. Two positive bovine sera control samples, one negative bovine sera control sample and a single non-serum control sample were included on each plate. The presence of *Neospora* spp. antibodies by IFAT was carried out according to http://www.sciencedirect.com/science/article/pii/S0304401715004021, Fish et al. (2007) by using FITC-labelled rabbit anti-dog, anti-sheep, anti-bovine, anti-deer and anti-goat secondary antibodies at a concentration of 1:60 for canids and 1:80 for mouflon, *Bovinae* species, *Cervidae* species and Nubian ibex. The highest dilution at which the whole parasites showed fluorescence was considered as the endpoint titre.

MAT for *Besnoitia* spp. was performed as previously described (Waap et al., 2011) with a few modifications: the initial serum dilutions were 1:100 and 1:200, and tachyzoites were resuspended to a final concentration of 40,000 tachyzoites/μL. A well-coded panel of 125 ruminant sera from cattle (n = 42 seropositive and n = 39 seronegative) (Gutiérrez-Expósito et al., 2017a), 35 from caribou (n = 15 seropositive and n = 20 seronegative) (Gutiérrez-Expósito et al., 2012) and 5 from sheep (n = 5 seronegative) (Gutiérrez-Expósito et al., 2017b) tested by Western blot were used to standardize an *in-house* MAT. Two samples from seropositive animals with dermal cysts and two samples that were seronegative by IFAT and Western blot from a non-endemic area were included in each plate. The dilution with the best values of sensitivity (Se) and specificity (Sp) was selected as a cut off.

Neospora caninum and *B. besnoiti* tachyzoites were processed and Western blots were carried out under reducing and non-reducing conditions as previously described (Alvarez-García et al., 2002; García-Lunar et al., 2013). Tachyzoites were exposed to sera from positive-IFAT and/or MAT animals using a 1:20 dilution and a second incubation step with Protein A–Peroxidase-labelled (P8651, Sigma) diluted at 1:200 was used for carnivorous species (golden jackal and red fox). Rabbit peroxidase-labelled anti-deer IgG (H + L) antibody conjugate (04-31-06 KPL, Gaithersburg, MD, USA) diluted at 1:200 was used for *Cervidae* sera (Persian fallow deer and roe deer), and protein G (Recombinant-Peroxidase Labeled, Sigma®), at a 1:500 dilution was used for *Bovidae* sera (addax, Arabian oryx, mouflon, mountain gazelle, Nubian ibex, scimitar horned oryx and water buffalo). For the *Neospora* spp.-based Western blot, the presence of the immunodominant 17–18 kDa antigen was the criterion for a positive result (Álvarez-García et al., 2002), whereas for the *Besnoitia* spp.-based Western blot, the criterion described by García-Lunar et al. (2013) was considered as a positive result.

Neospora spp. antibodies were found in golden jackal, red fox, addax, Arabian oryx, Persian fallow deer, mouflon, mountain gazelle, Nubian ibex, scimitar horned oryx and water buffalo by IFAT and/or MAT (Table 1). A cut-off of 1:200 was selected for both tests as only four out of 26 sera with an IFAT titre of 1:100 were confirmed by Western blot (data not shown) and none of the sera with a MAT titre of 1:100 (n = 157, data not shown) could be confirmed by IFAT. Specifically, 52 samples were seropositive by both serological techniques, and 141 were seropositive only by MAT. The highest antibody levels