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Molecular assessment of *Neospora caninum* and *Toxoplasma gondii* in hooded crows (*Corvus cornix*) in Tehran, Iran

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ABSTRACT

Neospora caninum and *Toxoplasma gondii* are two closely related protozoan parasites that have been detected from various species of bird hosts. However, little is known about the prevalence of *N. caninum* and *T. gondii* in crows. Hence, we examined the molecular frequency of *N. caninum* and *T. gondii* in the brain samples of hooded crows (*Corvus cornix*) that collected from different public parks of Tehran, Iran by nested-PCR method. We used the primers targeting the *Nc*5 and *GRA*6 genes for detection of *N. caninum* and *T. gondii*, respectively. From a total of 55 brain samples, 5 (9.9%) and 9 (16.36%) samples were positive for *N. caninum* and *T. gondii*, respectively. Sequencing of a *N. caninum* isolate revealed 95%–100% identity with the deposited *N. caninum* in GenBank. Genotyping of *T. gondii* isolates by PCR-RFLP analysis of the GRA6 gene revealed type III genotype in 8 isolates. The results of this study indicate that hooded crows may have a putative role in transmission of *N. caninum* and *T. gondii* to canines and felines definitive hosts, respectively.

1. Introduction

Neospora caninum and *Toxoplasma gondii* are two closely related cyst-forming apicomplexan parasites with a wide variety of intermediate bird hosts [1]. Canines and felines are definitive hosts of *N. caninum* and *T. gondii*, respectively, whereas many warm-blooded animals, including birds, serve as intermediated hosts [2,3]. Birds can be infected with *N. caninum* and *T. gondii* through ingestion of oocysts from the contaminated environments by canine and feline feces, respectively [2,3]. Corvids are widespread synanthropic birds that feed on various nutritional sources, such as carrion, small rodents and birds. Hence, corvids can also be infected by ingestion of cysts in muscle and brain of infected animals or carrions. On the other hand, infected birds and corvids might be the sources of infections for felines and canines definitive hosts of the parasites (Fig. 1) [2,3]. Until now, *N. caninum* was detected in different bird species such as chicken (*Gallus domesticus*) [4], sparrow (*Passer domesticus*) [5], magpies (*Pica pica*) and buzzard (*Buteo buteo*) [6]. *T. gondii* was also detected in a wide range of wild and domestic birds [7,8]. Moreover, these parasites were detected in several corvid species in several countries (summarized in Table 1). Nevertheless, little is known about the prevalence of *N. caninum* and *T. gondii* in crows. Hence, the aim of this study was determination of the presence of *N. caninum* and *T. gondii* in hooded crows (*Corvus cornix*) by molecular methods in Tehran, Iran.

2. Materials and methods

2.1. Sample collection and DNA extraction

A total 55 dead hooded crows (*Corvus cornix*) were collected by sweepers in different public parks of Tehran. The whole brain of each bird was obtained and kept on −20 °C until use. Individual brain samples of the crows were homogenized and DNA was extracted using a phenol–chloroform extraction method as previously described [5].

2.2. Molecular detection

Molecular diagnosis was performed by nested-PCR method using primers targeting the *Nc*5 and *GRA*6 genes for N. caninum and T. gondii, respectively. The primers and cycling conditions are shown in the Tables 2 and 3. In each reaction, a positive and a negative control were included. We used double distilled water for negative control and DNAs of the Nc5 strain of *N. caninum* and RH strain of *T. gondii* for positive control. PCR products were electrophoresed in 1.5% agarose gels and visualized under UV transillumination.

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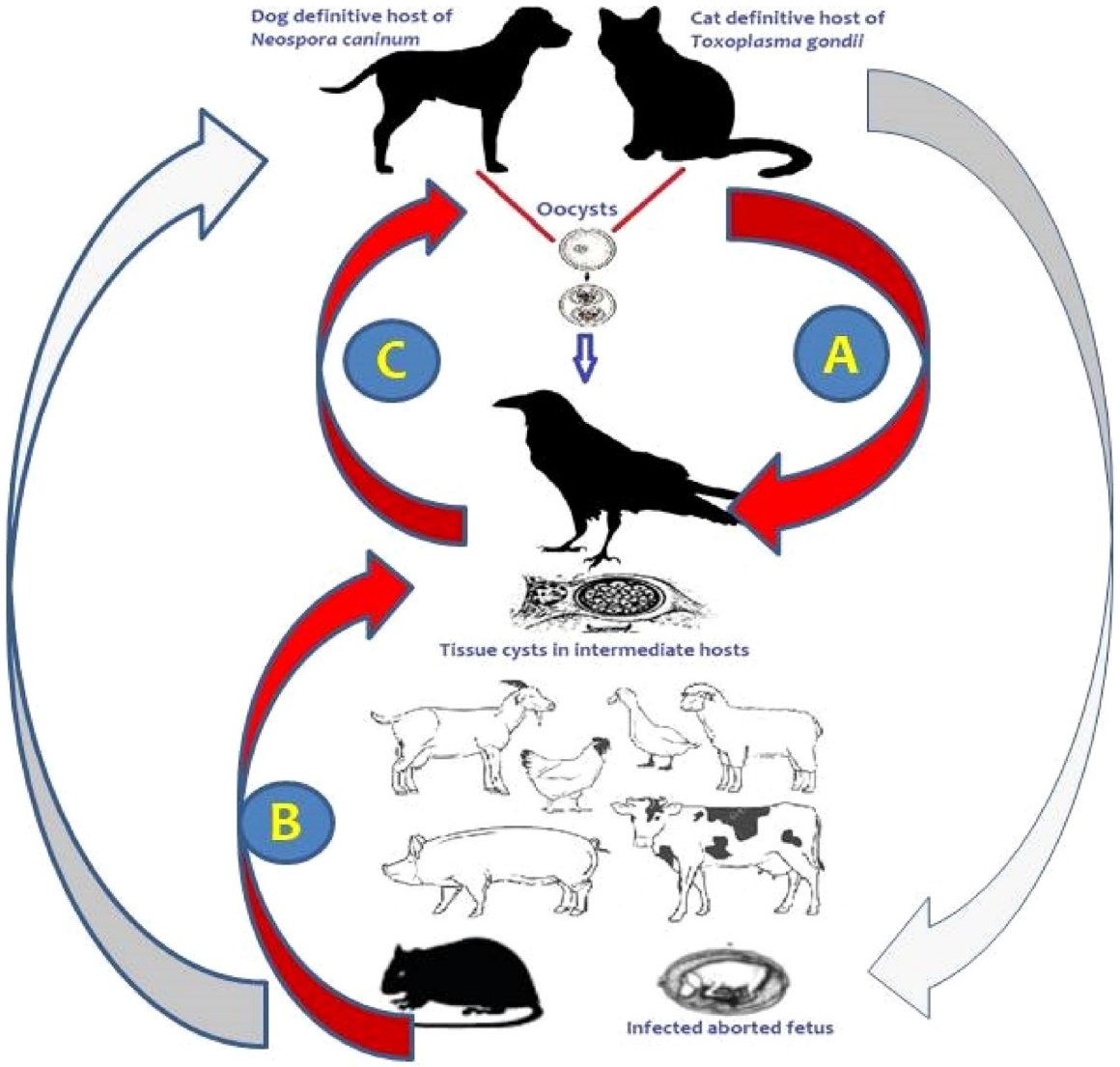

Fig. 1. Potential roles of corvides in maintenance and transmission of *N. caninum* and *T. gondii*. A) Infection of corvids through ingestion of oocysts from the contaminated environments. B) Infection of corvids via ingestion of tissue cysts from the infected carrions or aborted fetuses and placenta of infected animals. C) Transmission of the parasites from infected corvids to dog and cat definitive hosts of and *N. caninum* and *T. gondii*, respectively.

2.3. Nucleotide sequence analysis of a N. caninum isolates

A positive PCR product of *N. caninum* was extracted from the gel (Vivantis Gel Purification kit, Vivantis, Selangor Darul Ehsan, Malaysia) and sequenced in the forward and reverse directions [5,9]. The sequence was edited by with BioEdit software [10], aligned and compared with *Nc*5 partial sequences of *N. caninum* available in GenBank by ClustalW2 online tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/) [11].

2.4. Genotyping of T. gondii isolates by restriction fragment length polymorphism (RFLP)

Genotypes of *T. gondii* isolates were determined by PCR-RFLP method as previously described [12]. Briefly, nested-PCR products of *GRA*6 positive samples were digested by *Tru1*I (*Mse*I) restriction enzyme (Cat. No. ER0982, Thermo Fisher Scientific, USA), then the digested products were electrophoresed in 3% (w/v) agarose gel by Tris-acetate-EDTA (TAE) buffer and visualized under UV transillumination.

3. Results

N. caninum and *T. gondii* DNAs were detected in 5 (9.9%) and 9 (16.36%) out of 55 brain samples, respectively (Fig. 2a and b). We did not find any coinfection by *N. caninum* and *T. gondii* in the samples. A nucleotide sequence of *N. caninum* isolate with a length of 226 bp was submitted to the GenBank database with an accession number KR106185. Our sequence shared 95%–100% identity with *N. caninum* in GenBank. Interestingly, our sequence (KR106185) had 100 – 96% identity with *N. caninum* isolated from sparrows (KP702736, KR106182, KR106182, KR106181) and sheep (KR106181) in Tehran in our previous study [5,9] (Supplementary Table 1). From 9 *T. gondii* isolates, 8 samples completely digested by *Tru1*I (*Mse*I) restriction enzyme. The results identified that all eight isolates were similar to genotype III of *T. gondii* (Fig. 2c).

Table 1
A summary on the prevalence of *N. caninum* and *T. gondii* in several corvid species in different countries.

Year, location, and detection methods	Corvid species and number of studied	*N. caninum*	*T. gondii*	Ref
1976 California (USA), serology: IHA	*Corvus brachyrhynchos*, N = 74	–	14%	[17]
1981-1990 Czech Republic Mouse inoculation and tissue histopathology	*Corvus frugilegus*, N = 28	–	18%	[33]
1993-1999 Hawaii, USA, Serology: MAT	*Corvus hawaiiensis*, N = 27	–	18.%	[27]
2012 Spain, PCR	*Pica pica*, N = 33	6%	15%	[34]
2012 Spain Serology: MAT	*Corvus corax*, N = 113	35.8%	80.5%	[35]
2013 Israel Serology: MAT PCR	122 corvid species: *Corvus cornix, Corvus monedula* and *Corvus splendens*	–	Serology: 42.6%. PCR: 0.8%	[31]
2015 Israel, Serology: MAT and IFAT tests PCR	183 corvid species: *Corvus cornix, Corvus monedula* and *Corvus splendens*	Serology: 16.4%. PCR: 1.1%	–	[32]
2015 Tehran, Iran, Nested-PCR	*Corvus cornix*, N = 55	9.9%	16.3%	Current study

Common names: Hooded crows (*Corvus cornix*), Eurasian magpie (*Pica pica*), Common raven (*Corvus corax*), Rook (*Corvus frugilegus*), Jackdaw (*Corvus monedula*) and House crow (*Corvus splendens*), 'Alala (*Corvus hawaiiensis*), American crow (*Corvus brachyrhynchos*). MAT: modified agglutination test. IFAT: indirect fluorescent antibody test. IHA: indirect hemagglutination test.

4. Discussion

Corvids, including hooded crows (*Corvus cornix*) are synanthropic birds that widespread around the world and in Iran. According to previous studies, *N. caninum* and *T. gondii* were detected in different bird species such as chicken [13], sparrow [5,14] and their definitive hosts, including dog [15] and cat [13]. However, to our knowledge this is the first detection of *N. caninum* and *T. gondii* in crows in Iran. In the current study, DNA of *N. caninum* and *T. gondii* was detected in 9.9% and 16.36% of the crows in Tehran, Iran, respectively. In several studies *N. caninum* and *T. gondii* were detected in different corvid species (Table 3), although the majority of the studies were conducted on *T. gondii*. Finlay and Manwell (1956) was first detected *T. gondii* in the internal organs of American crows (*Corvus brachyrhynchos*) by mouse inoculation in New York (USA) [16]. Franti et al. (1976) investigated anti-*T. gondii* antibody in wild and domestic animals by indirect hemagglutination test (IHA) in northern California (USA). Franti et al. (1976) found that 3.5% of the investigated birds had specific antibodies against *T. gondii*, with the highest seroprevalence rates in crows [17]. In previous study, *N. caninum* DNA was detected in 3.6% of house sparrows (*Passer domesticus*) in Tehran [5]. *N. caninum* DNA was also detected in 3.68% of house sparrows (*Passer domesticus*) [5] and 2.1% of dogs (fecal samples) in Lorestan province (west of Iran) [18]. Antibody of *N. caninum* was detected in 17.33% of free ranging chickens (*Gallus domesticus*) in Tehran [19] and 9.4% of dogs in Tehran [20]. *T. gondii* have been detected in various bird species, including turkeys [21], chickens, ducks [13] and house sparrows [14]. *T. gondii* DNA also detected in 8.7% of soil samples in parks and public places in Tehran [22].

T. gondii has three main genotypes, including type I, II, and III, which show some differences in epidemiology and virulence patterns [23,24]. Type III genotype is the most prevalent *T. gondii* type in birds [23,24]. Also, type III is the most reported genotype of *T. gondii* in different hosts in Iran [13,25,26]. Hence our results are consistent with other reports in Iran.

Toxoplasmosis can cause mortality in several bird hosts [7], including crow [27]. Although experimental studies of neosporosis in some birds such as pigeons (*Columba livia*) [28] and chicken (*Gallus domesticus*) [29] revealed that the infection causes pathological lesions, the role of neosporosis in the mortality of birds is uncertain [30].

Corvids can get the infection through ingestion of tissue cysts from infected carrions or aborted fetuse and placenta of infected animals (Fig. 1). As corvids feed on various nutritional sources, including carrion, small birds and rodents which could be contaminated with N. caninum and T. gondii tissue cysts [31,32], it is plausible that the infection could be related to tissue cysts. Moreover, corvids could be a good sentinel for environmental contamination by these parasites.

In conclusion, this study demonstrated that hooded crows retain *N. caninum* and *T. gondii* in their tissues, and may have a putative role in transmission of the parasites to canines and felines definitive hosts.

Table 3
PCR conditions of the *Nc5* and *GRA6* genes.

Reaction stages	Nc5 [9]		GRA6 [38]	
Initial denaturation	5 min, 94 °C	1 Cycle	5 min, 94 °C	1 Cycle
Denaturation	40 s, 94 °C	40 Cycles	30 s, 94 °C	35 Cycles
Annealing	**PCR1:** 40 s, **62 °C** **Nested:** 40 s, **56 °C**		**PCR1:** 30 s, **58 °C** **Nested:** 30 s, **57.5 °C**	
Extension	40 s, 72 °C		30 s, 72 °C	
Final extension	10 min, 72 °C	1 Cycle	5 min, 72 °C	1 Cycle

Table 2
N. caninum and *T. gondii* specific primers targeting the *Nc5* and *GRA6* genes.

Markers	External primers (5′–3′)	Internal primers (5′–3′)	Ref
Nc5	F:CCCAGTGCGTCCAATCCTGTAAC R:CTCGCCAGTCCAACCTACGTCTTCT	F:CAGTCAACCTACGTCTTCT R:GGGTGAACCGAGGGAGTTG	[36,37]
GRA6	F:ATTTGTGTTTCCGAGCAGGT R: GCACCTTCGCTTGTGGTT	F:TTTCCGAGCAGGTGACCT R:CGCCGAAGAGTTGACATAG	[12]

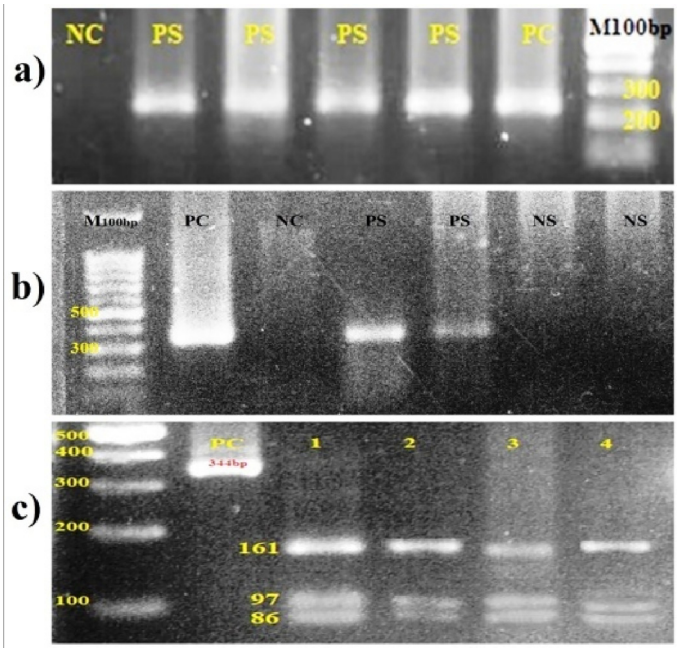

Fig. 2. PCR products of the *N. caninum* (a) and *T. gondii* (b) samples. RFLP patterns of *T. gondii* positive samples that matched to genotype III of *T. gondii* (c). PC: Positive control; NC: Negative control; PS: positive samples; NS: Negative samples.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cimid.2018.06.008.

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