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Detection and genotyping of *Toxoplasma gondii* strains isolated from birds in the southwest of Iran

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Abstract 1. The aim of this work was to determine the frequency of occurrence of *Toxoplasma gondii* and genetically analyse isolates from a number of avian hosts in the southwest of Iran (Khuzestan province). The frequency of *T. gondii* was determined in free-range chickens (*Gallus domesticus*), sparrows (*Passer domesticus*), pigeons (*Columba livia*) and starlings (*Sturnus vulgaris*).
2. Isolates obtained from *Toxoplasma*-infected birds were subjected to molecular typing by PCR-restriction fragment length polymorphism (RFLP) with sequence analysis of the GRA6 gene.
3. The results showed that 41 (16·5%) of 241 samples of avian tissue were infected with *T. gondii*. Sparrows were most frequently infected (17 out of 64).
4. Analysis of the GRA6 gene by PCR-RFLP and DNA sequencing revealed Type II and III *T. gondii* were the predominant lineage, accounting for 19·5% and 80·5% of the isolates, respectively.
5. It was concluded that the use of this PCR test facilitated the diagnosis of *T. gondii* in avian hosts and the GRA6 PCR-RFLP method clearly differentiated between the three different *T. gondii* lineages. This study showed a higher prevalence of type III compared with type II *T. gondii* in infected avian hosts in southwestern Iran.

INTRODUCTION

Toxoplasmosis is a widespread zoonosis caused by the obligate intracellular protozoan parasite, *Toxoplasma gondii*. Many warm-blooded vertebrates, including humans, birds, livestock, and marine mammals can be affected by toxoplasmosis (Dubey, 2010). The most common way for humans to become infected is by ingesting tissue cysts present in undercooked meat or by accidentally ingesting oocysts (Kijlstra and Jongert, 2008). Toxoplasmosis continues to be a public health problem in Iran where about 50% of the human population has been exposed to this parasite (Assmar *et al*., 1997).

Only a small number of exposed adults develop clinical signs of the disease. However, in human infants *T. gondii* infection causes various clinical signs including mental retardation, loss of vision and jaundice. In addition, toxoplasmosis can be an important cause of morbidity and mortality in immunosuppressed individuals (Montoya and Liesenfeld, 2004; López-Castillo and de-la-Torre, 2011). The genus *T. gondii* has been classified into three lineages: types I, II and III, based on virulence in mice and biological differences (Howe *et al*., 1997; Ferreira *et al*., 2004; Ferreira *et al*., 2006; Sibley and Ajioka, 2008). With the Type I strain, there is the risk of transplacentary transmission with increased severity of infection in developing foetuses, whereas Types II and III strains cause chronic infection and production of tissue cysts in mice (Howe *et al*., 1997).

Isolates of *T. gondii* have been genetically characterised in different parts of the world including Iran (Ajzenberg *et al*., 2005; Zia-Ali *et al*., 2005, 2007; Velmurugan *et al*., 2008; Soares *et al*., 2011), however, more isolates

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from different geographical locations would be valuable (Ajzenberg *et al.*, 2004).

Despite a high prevalence of *T. gondii* in avian hosts (Ghorbani *et al.*, 1990; Zia-Ali *et al.*, 2005; Asgari *et al.*, 2009), there has been no report on the prevalence and genetic characterization of *Toxoplasma* isolates from the southwest of Iran.

The aim of this study was the diagnosis and genotypic characterization of *T. gondii* isolates in infected birds destined for human consumption.

MATERIALS AND METHODS

Study area

Khuzestan province is located in the south west of Iran with an area of about 64055 km^2 and located at 31·3273° N and 48·6940° E. Ahvaz city is located in the middle of the province and on the banks of the Karun River. It has a desert climate with hot summers and short, mild winters. The city has an average elevation of 20 m above sea level with an average annual rainfall about 230 mm.

Sample collection

A total of 249 samples from 103 free-range chickens, 43 pigeons, 64 sparrows and 39 starlings that were purchased from different sources in cities of Khuzestan Province from March to August 2011. All birds were killed by cervical dislocation; hearts and brains were removed and transferred to the laboratory under refrigeration.

DNA extraction and molecular diagnosis of infected birds

Brains and hearts of each sample were homogenized in phosphate-buffered saline at pH 7·2. DNA was extracted using a standard extraction procedure with the QIA DNeasy blood and tissue kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions and DNA concentration was measured at 260 nm using a spectrophotometer.

For direct diagnosis of infected birds, primers were chosen from the B1 gene sequences as Tg1 (5′-AAAAATGTGGGAATGAAAGAG-3′ and Tg2 (5′-ACGAATCAACGGAACTGTAAT-3′) as described by Jalal *et al.* (2004). Amplifications were carried out using an AccuPower PCR PreMix kit (Bioneer Co., Daejeon, S. Korea). The PCR was performed by adding 5 µl of each DNA template and 25 pmol of each primer to provide a final volume of 20 µl. The PCR mix was composed of 1 unit of Taq DNA polymerase, 10 mM Tris-HCl, 30 mM KCl, 1·5 mM $MgCl_2$, and 250 mM of each dNTP. Each amplification run contained one positive control (DNA extracted from the RH strain) and a negative control. The thermocycler used (MyCycler; Bio-Rad, Hercules, CA, USA) was set to give 5 min at 94°C, followed by 35 cycles, each of 45 s at 94°C, 30 s at 50°C and 60 s at 72°C, and then a final extension for 60 s at 72°C. Electrophoresis was performed after adding a 5 µl sample of the PCR products to a 1·5% agarose gel. The gel was stained with ethidium bromide for 45 min at 100 V. Bands were visualised by ultraviolet transillumination (BioSystematica, Llandysul, Ceredigion, UK).

Amplification of GRA6 as a target for genetic polymorphism analysis

Genetic analysis of toxoplasma isolates was carried out using PCR-restriction fragment length polymorphism (RFLP) analysis of the GRA6 gene as described by Fazaeli *et al.* (2000). Two primers were designed from the GRA6 gene sequences; forward primer, 5′ GTAGCGTGCTTGTTGGCGAC-3′ and reverse primer, 5′-TACAAGACATAGAGTGCCCC-3′ which amplified an 800-bp DNA fragment of the target gene. The PCR was performed by adding 5 µl of each DNA template and 25 pmol of each primer to provide a final volume of 20 µl (AccuPower PCR PreMix kit from Bioneer Co., Daejeon, Korea). The target DNA was amplified using the following thermal conditions: 5 min at 94°C, followed by 35 cycles, each of 45 s at 94°C, 30 s at 50°C and 60 s at 72°C, and a final extension for 7 min at 72°C. All PCR products were digested for 4 h, with the restriction endonuclease *MseI* using buffers recommended by the manufacturer (Fermentas, Vilnius, Lithuania) in a total volume of 20 µl at 37°C. Restriction fragments of amplicons were electrophoresed using a 1·6% agarose gel at 100 V for 180 min and were stained with ethidium bromide and visualised by UV transillumination (BioSystematica, Llandysul, Ceredigion, UK).

Nucleotide sequences

DNA derived from infected birds was subjected to sequencing by MWG (Ebersberg, Germany) using the primers employed in the GRA6 PCR analysis. The data were analysed using Chromas software (http://www.technelysium.com.au/Chromas.htm) and compared with the sequence in the NCBI nucleotide gene bank (http://www.ncbi.nlm.nih.gov/BLAST/).

Ethical approval

All birds were handled using procedures recommended by the Animal Care Program, U.S. Department of Agriculture, USA (http://

Table. *The results of PCR analysis in organs of different avian hosts and characterization of Toxoplasma isolates based on GRA6 PCR-RFLP patterns in the southwest of Iran*

Species	No. tested	Positive No. (%)	PCR positive organ		RFLP patterns[1]	
			Heart (%)	Brain (%)	II (%)	III (%)
Free-range chicken (*Gallus domesticus*)	103	16 (15·5)	10 (62·5)	6 (37·5)	4 (9·7)	12 (29·2)
Sparrow (*Passer domesticus*)	64	17 (26·5)	13 (76·4)	4 (23·5)	1 (2·4)	16 (39)
Pigeon (*Columba livia*)	43	3 (6·9)	2 (66·6)	1 (33·3)	1 (2·4)	2 (4·8)
Starling (*Sturnus vulgaris*)	39	5 (12·8)	3 (60·0)	2 (40·0)	2 (4·8)	3 (7·3)
Total	249	41 (16·46)	28 (68·2)	13 (31·7)	8 (19·5)	33 (80·4)

[1]No occurrences of RFLP type I were detected

www.aphis.usda.gov/animal_welfare/downloads/awr/awr.pdf).

RESULTS

Molecular diagnosis of infected birds

The Tg1 and Tg2 primers amplified a 469-bp DNA fragment of the target B1 gene (data not shown). Analysis of the DNA samples showed that 41 (16·5%) samples were infected with *T. gondii*. *Toxoplasma* DNA was detected in 17 out of 64 sparrows, 16 out of 103 free-range chickens, 4 out of 39 starlings and only 2 out of 43 pigeons (Table).

Genotyping of *T. gondii* using PCR-RFLP and nucleotide sequences

All isolates positive for the B1 gene showed a 800 bp band (Figure A). After the PCR products were digested with the *MseI* restriction enzyme, analysis of the isolates based on GRA6 PCR-RFLP pattern (Figure B) showed that 80·4% were type III and only 19·5% were type II. No type I isolates were detected. In the 16 infected free-range chickens, type III was most prevalent (12 cases). Only one sparrow was infected with type II (Table).

The GRA6 gene fragments from five randomly selected isolates were sequenced and submitted in the GenBank database with accession numbers: AB703305·1, AB703303·1, AB703299·1, AB703306·1 and AB703304·1.

DISCUSSION

In this study, we used a PCR assay for direct diagnosis and identification of *T. gondii* strains isolated from different avian hosts. Earlier studies carried out in Iran mostly used serological methods for diagnosis and demonstrated a high prevalence of *toxoplasma* antibodies (33% to 51%: Zia-Ali *et al.*, 2005, 2007; Asgari *et al.*, 2006, 2009). In contrast, the rate of infection in free-range chickens in southwestern Iran, reported here, was only 16·5%. The lower rate of toxoplasma infection using the PCR method is probably related to the presence of tissue cysts in the samples. Asgari *et al.* (2009) also reported that when using PCR analysis a number of tissue samples from seropositive birds were negative, suggesting that tissue cysts are probably not present in the seropositive samples.

The highest rate of infection was observed in sparrows and chickens. Although the house sparrow is relatively resistant to toxoplasmosis, they are frequently consumed by feline predators and these wild birds could be a source of infection for cats (Ruiz and Frenkel, 1980), so allowing the parasite life cycle to continue. It is likely that free-range chickens and sparrows are infected by feeding on ground contaminated with oocysts. By contrast, the lowest rate of infection was found in pigeons, probably because the pigeons were kept in cages and fed by humans.

Several different methods have been employed for genotyping *Toxoplasma* strains including antigen analysis (Bohne *et al.*, 1993), isoenzyme assays (Darde *et al.*, 1992), randomly amplified polymorphic DNA (RAPD), PCR detection (Ferreira *et al.*, 2004) and RFLP (Howe *et al.*, 1997; Ferreira *et al.*, 2006). In the present study, a PCR-RFLP method based on the nucleotide polymorphisms of the GRA6 coding region was used that was previously described by Fazaeli *et al.* (2000).

Analysis of the isolates from different birds indicates that these avian isolates were avirulent and belonged to Types II and III. This accords with earlier reports and confirms that type III strains are associated with the majority of cases of avian toxoplasmosis (Dubey *et al.*, 2003, Zia-Ali *et al.*, 2005, 2007). Howe and Sibley (1995) reported that type III was more common in animals than in humans. A similar study from Egypt reported that type III toxoplasma were predominant in free-range chickens (Dubey *et al.*, 2003). Therefore, comparable geographical and ecological conditions could be factors

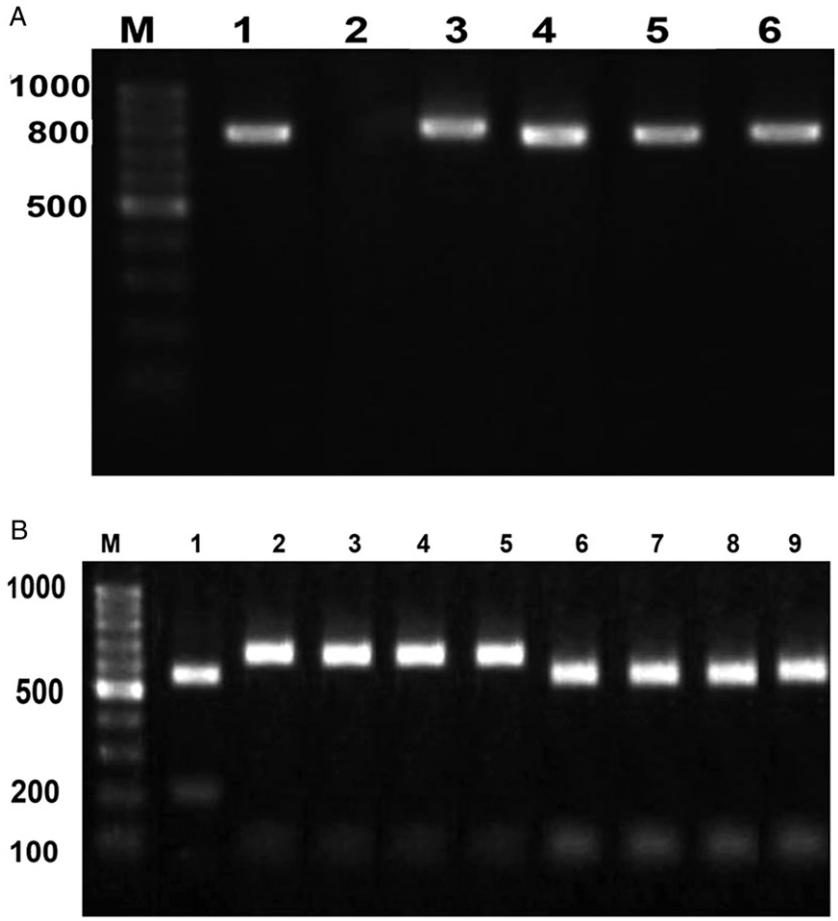

Figure. *(A) Result of PCR analysis of the GRA6 gene from Toxoplasma gondii-infected samples. M: 100 bp molecular size marker. Lane 1: Positive control RH strain. Lane 2: Negative control. Lanes 3-6: bands of 800 bp from Toxoplasma-infected free-ranging chicken, sparrow, pigeon and starling respectively. (B) The MseI restriction fragments of the GRA6 gene. M: 100 bp molecular size marker. Lane 1: positive control type1 (RH strain). Lanes 2-5: strain type II from Toxoplasma-infected free-ranging chicken, sparrow, pigeon and starling respectively. Lanes 6-9: strain type III from Toxoplasma-infected free-ranging chicken, sparrow, pigeon and starling, respectively.*

influencing the *Toxoplasma* genotype in birds in southwestern Iran and Egypt. The frequency of type II in this study was 19·6%, consistent with earlier studies, and confirms that type II strains are most frequently associated with cases of toxoplasmosis (Dubey *et al*., 2002; Sreekumar *et al*., 2003).

No type I isolates were detected in this study. Similar results have been obtained with free-range chickens in United States (Dubey *et al.*, 2003) and India (Sreekumar *et al.*, 2003). This contrasts with reports from Brazil where a considerable number the isolates were found to be Type I (Dubey *et al*., 2002; Zia-Ali *et al.*, 2007).

In conclusion, the present study indicates that free-range chickens, sparrows, pigeons and starlings could be useful indicator species for the presence of soil contamination with *T. gondii* oocysts. Based on life style and dietary habits in southwestern Iran, meat from these avian hosts probably provides an important source of infection for humans.

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