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Short Communication

Molecular detection and genetic characterization of *Toxoplasma gondii* in farmed raccoon dogs (*Nyctereutes procyonoides*) in Shandong province, eastern China

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ABSTRACT

Toxoplasma gondii is a successful opportunistic parasite, affecting a wide range of vertebrate animals and humans. Genetic diversity of *T. gondii* in raccoon dogs (*Nyctereutes procyonoides*) is of great importance to understand the transmission of *T. gondii* in the environment. However, no information is available about the distribution of genetic diversity of *T. gondii* infection in raccoon dogs. This study was conducted to estimate the prevalence and genetic characterization of *T. gondii* from raccoon dogs in Shandong province, eastern China. A total of 314 brain tissue samples of raccoon dogs were collected and genomic DNA was extracted and assayed for *T. gondii* infection using semi-nested PCR targeting B1 gene. The positive DNA samples were typed at 10 genetic markers (SAG1, SAG2(5′ + 3′ SAG2, alter.SAG2), SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico) by multiplex multilocus nested polymerase chain reaction-restriction fragment length polymorphism (Mn- PCR-RFLP) technology. Thirty-five (11.15%) of 314 DNA samples were detected positive. Only six samples were completely typed at all genetic loci, and these samples represented ToxoDB genotype#9. Two sample were typed at 9 genetic loci and one was typed at 8 genetic loci, all of them represented Type I. To our knowledge, this is the first report of genetic characterization of *T. gondii* in raccoon dogs in China. These results revealed the existence of genetic diversity of *T. gondii* in raccoon dogs in China. These data provided base-line information for controlling *T. gondii* infection in raccoon dogs.

1. Introduction

Toxoplasma gondii is the most successful opportunistic intracellular pathogen, with a complex life cycle involving sexual propagation in cats (and felid) and asexual replication in virtually all warm-blooded animals and humans, including raccoon dogs (Dubey, 2010; Liu et al., 2015). Cats as definitive hosts of *T. gondii* can excrete infectious oocysts, and other animals are infected mainly through ingesting food or water contaminated with *T. gondii* cysts or oocysts (Choi et al., 1997; Aramini et al., 1999; Sibley and Ajioka, 2008). Infection with *T. gondii* may cause devastating diseases in immunocompromised individuals and pregnant women (Ryan et al., 1993; Michel et al., 1994; Dawis et al., 2002; Findal et al., 2015; Vijaykumar et al., 2016). Approximately one-third of the world population and 7.9% of Chinese people were infected with *T. gondii* (Dubey, 2010; Zhou et al., 2011).

Although isolates of *T. gondii* have been considered to represent a single species, but genetic diversity exists among *T. gondii* isolates from different hosts and geographical localities (Lehmann et al., 2006; Su et al., 2010; Shwab et al., 2014). The dominant strains of *T. gondii* in North America and Europe are mainly classified into four clonal lineages (Types I, II, III and 12), whereas, *T. gondii* isolates in South America and Africa have more genetic diversity (Shwab et al., 2014; Cong et al., 2015). There is a potential correlation between the genotypes of *T. gondii* and the virulence of *T. gondii*, for example, compared with type II and III lineages, Type I lineages are more virulent in mice even though the differences at the genomic level among the three lineages are less than 1% (Sibley and Boothroyd, 1992; Saeij et al., 2005; Shwab et al., 2014; Jensen et al., 2015). Besides, different

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genotypes of *T. gondii* are different in sensitivity to drugs (Meneceur et al., 2008). Therefore, it is very necessary to investigate the distribution of *T. gondii* genotypes all over the world, especially in China where limited information is available. Data on *T. gondii* genotypes have been reported in white yaks (Qin et al., 2015), geese (Rong et al., 2014), pets (Cong et al., 2014), cancer patients (Cong et al., 2015) and several kinds of wild animals in China (Qin et al., 2014; Zhang et al., 2015; Zhang et al., 2016), However, the information on *T. gondii* genotypes is not available in raccoon dogs (*Nyctereutes procyonoides*) in China, which is one of the most important economic animals. Raccoon dogs are also an important source of *T. gondii* infection for other animals and humans. Therefore, the objective of the present study was to investigate the *T. gondii* prevalence and genetically characterize *T. gondii* isolates in raccoon dogs to provide base-line information for controlling *T. gondii* infection in raccoon dogs in China.

2. Methods

2.1. Sample collection

This study was approved before its commencement by the Animal Administration and Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences. Raccoon dogs were handled in accordance with the requirements of the Animal Ethics Procedures and Guidelines of the People's Republic of China. A total of 314 brain tissue samples of raccoon dogs were collected from slaughtered raccoon dogs in Shandong province between October and November in 2015. All samples were grinded in liquid nitrogen and stored at −20 °C until DNA extraction.

2.2. Genomic DNA extraction of T. gondii isolates

Genomic DNA was extracted directly from brain tissue samples by using a commercial DNA extraction kit (TianGen™, Beijing, China). Briefly, 30 mg mashed samples were treated with sodium dodecyl sulphate/proteinase K at 56 °C for 6 h in a thermostat water bath. Subsequently, column purification was performed according to the manufacturer's recommendations, and DNA samples were eluted into 80 μl elution buffer. Genomic DNA was stored at −20 °C until further analysis.

2.3. Genetic characterization of T. gondii isolates

T. gondii B1 gene was amplified from all of the DNA samples to detect possible infection by a semi-nested PCR according to previous studies (Hill et al., 2006; Zheng et al., 2016). *T. gondii*-positive DNA samples were selected for further genetic characterization. Genotyping of *T. gondii*-positive DNA samples was performed using 10 genetic markers (SAG1, SAG2(5′ + 3′ SAG2, alter.SAG2), SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico) using multiplex multilocus nested PCR restriction fragment length polymorphism analysis (Mn-PCR-RFLP) as described previously (Su et al., 2006; 2010; Rajendran et al., 2012; Cong et al., 2015; Zheng et al., 2016). DNA samples of nine reference *T. gondii* strains (GT1, PTG, CTG, MAS, TgCgCa1, TgCatBr5, TgWtdSc40, TgCatBr64 and TgRsCr1) were included as positive controls (Table 1). The PCR reaction (25 μl) was composed of 1 × PCR buffer, 0.2 mM of each primer, 200 μM dNTPs, 2 mM $MgCl_2$, 0.2 U of r*Taq* DNA polymerase (TAKARA, Japan), and 1 μl DNA template. The cycling conditions were 95 °C for 5 min to activate the r*Taq* DNA polymerase, then 30 cycles of 95 °C for 30 s, 55 °C for 60 s and 72 °C for 90 s. Then, one microliter of the first Multiplex PCR amplicon served as template for nested PCR with internal primers for each marker, respectively. The nested PCR was carried out with an uniform annealing temperature at 60 °C for 60 s for all the markers except for the Apico (55 °C for 60 s) marker.

The second PCR products were digested with different restriction enzymes at suitable temperatures for 1.5 h. The restriction fragments were separated by 2.5%-3.0% agarose gel containing GoldView™ (Solarbio, China), visualized under ultraviolet light. The enzyme digestion fragment size of the sample was compared with the standard strain to determine the genotype at each locus. The results were compared and matched with those identified RFLP genotypes listed in ToxoDB genotyping database (www.toxodb.org). The primers used in this study are listed in Table S1.

3. Results and discussion

Among the 314 examined brain tissue samples of raccoon dogs, thirty-five (11.15%) samples were detected to be positive for the *T. gondii* B1 gene by semi-nested PCR, which was lower than the 18.3% (n = 20) in main-land raccoon dogs in Zoo in Japan by latex fixation tests (Murasugi et al., 1996), and 60% (n = 6) in Poland by PCR (Gorecki et al., 2012), but higher than the 3.3% (n = 1) in suburban areas in Japan (Neagari et al., 1998). Regrettably, very limited information was available to contrast and discuss the *T. gondii* prevalence in raccoon dogs in different countries. Furthermore, no information is available prior to the present study on the genotypes of *T. gondii* isolates in raccoon dogs.

Due to low DNA concentration, of the 35 positive samples, only six samples were completely typed at all genetic loci, two samples were typed at 9 genetic loci, and one was typed at 8 genetic loci, showing two genotypes (ToxoDB#9 and Type I) (Table 1). Six (66.67%) samples were identified as ToxoDB genotype#9, namely the so-called Chinese I isolate, which is the predominant genotype found in other animals and humans in China (Zhou et al., 2009; Qian et al., 2012; Jiang et al., 2014; Cong et al., 2015, 2016). Type I (ToxoDB genotype#10) was identified from three samples in this study, which was also widely prevalent in China (Zhou et al., 2009; Qin et al., 2014; Zhang et al., 2014; Cong et al., 2015; Miao et al., 2015). Remarkably, the genotypes of *T. gondii* in raccoon dogs were in accordance with cancer patients in Shangdong province, China (Cong et al., 2015), revealing that cross infection could be existed between animals and humans in this province. Some studies in North America and Europe showed that most human cases of *T. gondii* infection were due to type II strains that had higher capacity to produce cysts in animal models (Howe and Sibley, 1995; Howe et al., 1997; Ajzenberg et al., 2002), which was different from the situation in China that ToxoDB#9 and Type I were the dominant genotypes in cancer patients.

Raccoon dogs have very high economic value because its wool skin is a good raw material for making a coat, collar, hat and leather and fur products of the mattress. Raccoon dog meat is not only delicious food, but also can be used as medicine. Raccoon dog gallbladder (juice) after drying can replace bear gall medicine, and has been used for the treatment of gastrointestinal disease and epilepsy in children. Therefore, *T. gondii* prevalence in raccoon dogs should be monitored regularly due to they may become a source of human infection with *T. gondii* by ingestion of undercooked or raw meat of infected raccoon dogs. Compared with other animals (such as pigs, chicken, cattle, sheep and dogs), although raccoon dog meat makes a small part of the human food, but it should not be ignored as a potential source of *T. gondii* infection in humans in China.

4. Conclusions

The present study revealed, for the first time, a high (11.15%) *T. gondii* prevalence in raccoon dogs by PCR in Shandong Province, China. Two genotypes (ToxoDB genotype#9 and Type I) were firstly identified in raccoon dogs, which provided base-line data for controlling *T. gondii* infection in raccoon dogs.