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Research paper

Prevalence, risk factors and genetic characterization of *Toxoplasma gondii* in sick pigs and stray cats in Jiangsu Province, eastern China

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ABSTRACT

Toxoplasma gondii is an obligate intracellular parasitic protozoan with a worldwide distribution. The parasites in edible tissues of pigs and oocysts from cats are the major sources of *T. gondii* infection in humans. However, there are no data from sick pigs in veterinary clinics or from stray cats in Jiangsu Province, eastern China. In total, biological samples from 141 sick pigs and 64 stray cats were collected from this region. The rate of *T. gondii* infection in sick pigs was 46.81% using a polymerase chain reaction (PCR), and the overall prevalence of toxoplasmosis in stray cats was 34.38% by PCR and an enzyme-linked immunosorbent assay (ELISA). *T. gondii* was significantly more prevalent in lungs and heart than in liver and spleen ($P < 0.05$). Age and geographic region were considered to be the main risk factors associated with *T. gondii* infection in these pigs. The DNA samples from 17 sick pigs and seven stray cats, were successfully genotyped by multilocus PCR-restriction fragment length polymorphism (PCR-RFLP) with 10 genetic markers [*SAG1*, *SAG2* (5'-3'*SAG2*, alt. *SAG2*), *SAG3*, *GRA6*, *PK1*, *c22–8*, *c29–2*, *BTUB*, *L358* and Apico]. Six distinct genotypes were found, which were designated ToxoDB PCR-RFLP genotypes #9 (Chinese I), #10 (Type I), #213, and #89, and New 1 and New 2. Chinese I is the most prevalent *T. gondii* genotype in this region. The two new genotypes (designated New 1 and New 2) are reported and the ToxoDB PCR-RFLP genotype #89 is found for the first time in China. Such information will be useful for the prevention, diagnosis and treatment of porcine toxoplasmosis in Jiangsu Province, eastern China.

1. Introduction

Toxoplasmosis is a prevalent zoonotic disease with a worldwide distribution and occurs in all warm-blooded animals, including humans. It is caused by the protozoan *Toxoplasma gondii* and can trigger serious disease in many species. In humans, infection results mainly from the ingestion of undercooked meat containing *T. gondii* cysts, or by drinking water contaminated with oocysts from feces of infected cats (Taylor et al., 2007). Severe disease can occur in immunocompromised individuals (Montoya and Liesenfeld, 2004) and, in pregnant women who become acutely infected, the parasite can also cause severe fetal abnormalities or fetal death (Hill et al., 2005; Tenter et al., 2000). Recently, toxoplasmosis has been associated with a range of neurological and psychiatric disorders in humans (Lafferty, 2006; McAllister, 2005; Palmer, 2007; Torrey et al., 2007, 2015). In addition, *T. gondii* infection can also cause severe damage to livestock, inducing the acute onset of toxoplasmosis and death in pigs and abortion in sheep, resulting in significant economic loss.

The pig (*Sus scrofa*) is economically valuable in terms of meat production worldwide. *T. gondii* infections can either cause death in pigs, or can be asymptomatic and self-limiting as a result of differences in parasite number and virulence among strains; however, the persistence of the parasite in edible tissues of pigs (Dubey, 2010; Hill and Dubey, 2013) is a major source of *T. gondii* infection in humans as a result of the ingestion of porcine meat containing tissue cysts (Schluter et al., 2014). Felids are also important in the epidemiology of toxoplasmosis because they are the only hosts that can excrete the environmentally resistant oocysts into the environment. The prevalence of domestic cats (*Felis catus*) as both pets and strays throughout the world (Lepczyk et al., 2010) render them the main source of *T. gondii* infection for humans and other intermediate hosts. The rate of *T. gondii* infection in cats varies according to age and 'lifestyle' (Dubey, 1973), with stray

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cats having a higher positive rate of *T. gondii* infection compared with those kept as pets (Nutter et al., 2004).

T. gondii is the only recognized species in the genus *Toxoplasma*. However, distinct, genetically diverse populations of *T. gondii* have evolved in different geographic regions, as revealed by polymerase chain reaction -restriction fragment length polymorphism (PCR–RFLP) analyses, multiple enzyme electrophoresis analyses, and microsatellite methods (Lehmann et al., 2006; Su et al., 2010). Molecular epidemiology studies revealed that three genotypes (Types I, II, and III) predominate in North America and Europe (Howe and Sibley, 1995), whereas other diverse genotypes are found in other parts of the world (Sibley and Ajioka, 2008); for example: ToxoDB PCR-RFLP genotypes #1 (Type II), #2 (Type III), #3 (Type II variant), and #10 (Type I) occur globally (Shwab et al., 2014); genotypes #2 and #3 dominate in Africa; ToxoDB #9 (Chinese I) and #10 are prevalent in Asia; ToxoDB #1, #2, and #3 are prevalent in Europe; ToxoDB #1, #2, #3, #4 and #5 dominate in North America (#4 and #5 are collectively known as Type 12). To date, 12 genotypes have been identified from humans and animals in China, with genotype Chinese I being the most common on mainland China (Wang et al., 2016).

Swine toxoplasmosis in China was first reported in 1977 in Jiangsu and Shanghai. Jiangsu Province is located on Yangtze River Delta on mainland China (Fig. 1B; from 116° 18′ to 121° 57′ and 30° 45′ to 35° 20′). It has a warm temperate to subtropical transition climate with mild, moderate rainfall and four distinct seasons. It is one of the four most-densely populated provinces on mainland China, with a significant number of domestic cats in residential areas and stray cats around farms and in residential areas. Additionally, Jiangsu Province is an important center for pig farming, with approximately 29.78 million pigs slaughtered in 2015. Given the importance of cats to the transmission of toxoplasmosis and the significant negative effect that this disease has on pigs, as well as the risk of transmission to humans, there is a need to understand the epidemiology of both swine and feline toxoplasmosis in this region. Therefore, the present study was conducted to provide theoretical information for the clinical diagnosis, prevention, and control of toxoplasmosis in sick pigs and stray cats in Jiangsu Province, as well as to investigate the genotypes of *T. gondii* present in these animals. To our knowledge, this is the first report to document the genotypes of *T. gondii* in sick pigs and stray cats in Jiangsu Province.

2. Materials and methods

2.1. Ethics approval

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and the study was approved by the Animal Care and Use Committee of the College of Veterinary Medicine, Yangzhou University (Approval ID: SYXK [Su] 2012-0029). All animals were sacrificed under deep anesthesia using sodium pentobarbital when necessary; all efforts were made to minimize suffering.

2.2. T. gondii strains

RH strain, an international standard virulent strain, were provided by Yonghua Zhou from the Jiangsu Institute of Parasitic Diseases (Wuxi, China) and maintained in our laboratory. Genomic DNA of the ME49 strain was provided by Qijun Chen from the College of Animal Science & Veterinary Medicine, Shenyang Agricultural University (Shenyang, China).

2.3. Naturally infected animals

The 141 sick pigs used in the study were autopsied at the veterinary hospital of Yangzhou University (Yangzhou, China) from September 2011 to August 2016. All sick pigs were sourced from 14 regions of Jiangsu and Anhui Provinces, and the Shanghai Municipality in eastern China (Fig. 1A). Sick pigs were characterized by symptoms of poor mental state, fever, and dyspnea. Given that most of the pigs were dead (36 sick versus 105 dead pigs), it was not possible to collect serum samples to test for *T. gondii* antibodies. Therefore, only tissue samples were tested and analyzed for *T. gondii* infection. In total, 620 tissue samples, including liver, lungs, spleen, kidneys, lymph nodes and heart, were collected from 141 pigs ranging in age from < 30-days old to > 70-days old.

Sixty-four stray adult cats were collected from Yangzhou, Jiangsu Province or the surrounding areas from March 2013 to October 2015. All the stray cats, which were initially used for pharmacological experiments related to intestinal helminths, were provided by Shijin Bu from the College of Veterinary Medicine, Yangzhou University (Yangzhou, China). Following euthanasia, blood samples and tissues, including brain, muscle, lungs, heart, liver, spleen and lymph nodes, were collected. DNA samples were extracted from animal tissues and stored at −20 °C before use. Sera samples were also stored at −20 °C. All animal care and procedures were conducted according to the guidelines for animal use in toxicology. The study protocol was approved by the Animal Care and Use Committee of the College of Veterinary Medicine, Yangzhou University.

2.4. Detection of T. gondii by PCR

Genomic DNA samples of purified parasites and tissues of sick pigs and stray cats were extracted using a saturated phenol-chloroform extraction method. A PCR targeting the 529-bp repetitive sequence (AF146527) was established as previously described (Ellis, 1998) using specified primers (Homan et al., 2000). Each reaction comprised 1 × PCR Buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 0.2 mM dNTP, 0.4 μM of each primer, 0.5 U of *Taq* DNA polymerase, and 2 μL of template to a total volume of 50 μL. The conditions for amplification were as follows: 95 °C for 4 min, 35 cycles of 94 °C for 30 s, 58 °C for 40 s, and 72 °C for 1 min, and a final step of 72 °C for 10 min. The genomic DNA of the RH strain was set as the positive control, whereas the tissue DNA of a pig or cat, confirmed *T. gondii* infection-free by PCR, was the negative control for each reaction.

2.5. Serological examination

An ELISA for the examination of antibodies against *T. gondii* was performed on the feline sera using a commercial test kit (Shanghai Ding Biological Technology Co., Ltd. Shanghai, China) according to the manufacturer's recommendations. Stray cat sera with titers of 1:5 or higher were considered positive for *T. gondii* antibodies, with a cut-off of ≥0.4 OD units. Serum samples that gave unclear results were re-tested. The positive and negative control sera provided by the commercial kits were used in each test.

2.6. Genetic characterization of T. gondii isolates

Genotyping of *T. gondii* isolates was performed using multilocus PCR-RFLP with ten genetic markers as described previously (Su et al., 2010): *SAG1*, *SAG2* (5′–3′ *SAG2*, alt. *SAG2*), *SAG3*, *BTUB*, *GRA6*, *c22–8*, *c29–2*, *L358*, *PK1*, and Apico. Briefly, a multiplex PCR assay was performed based on the DNA template with mixed external primers in a total volume of 25-μL containing 1 × PCR buffer, 2.5 mM Mg^{2+}, 200 μM dNTP, 0.2 μM forward and reverse primers, 0.25 U *Taq* DNA polymerase, 1.5 μL DNA template, and ddH_2O. The conditions for amplification were as follows: 95 °C for 4 min, 35 cycles of 94 °C for 30 s, 55 °C for 1 min and 72 °C for 2 min, and a final step of 72 °C for 10 min. Multiplex products diluted (1:1) in water served as template DNA for nested PCR with internal primers for each marker. Next, 1 × PCR