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The prevalence of intestinal trichomonads in Chinese pigs

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ABSTRACT

Intestinal infection of pigs with trichomonads, (*Tritrichomonas suis*, *Tetratrichomonas buttreyi,* and *Pentatrichomonas hominis*) has been reported in many countries, such as the Philippines, Belgium, and the Czech Republic. However, the prevalence of trichomonads infection of swine in China has not yet been investigated. In this study, we used small subunit ribosomal RNA genes to detect the prevalence of *T. suis, T. buttreyi* and *P. hominis* among 158 fecal specimens from healthy pigs. Infection rates were 12.03%(19/158), 14.57% (23/158), and 24.05% (38/158) for *T. suis, T. buttreyi* and *P. hominis*, respectively. Molecular evolutionary analysis showed minor allelic variation in *T. buttreyi* from China compared to *T. buttreyi* isolated from other hosts in different parts of the world.

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1. Introduction

Infections from *Tritrichomonas* (a flagellated protozoa) are frequently reported in veterinary medicine (Felleisen, 1999). Trichomonads are anaerobic protists which harbor hydrogenosomes instead of mitochondria, and possess up to six flagella (Mostegl et al., 2011). These flagellated protozoa have a broad host range for vertebrates as well as invertebrates (Ibanez-Escribano et al., 2013). In swine, three species of trichomonads (*Tritrichomonas suis*, *Tritrichomonas rotunda,* and *Tetratrichomonas buttreyi*) have received much attention due to their wide-spread infections (Hibler et al., 1960). Recently, our laboratory identified infections of *Pentatrichomonas hominis* in pigs, and indicated the gastrointestinal tract as the main point of infection (Li et al., 2014).

T. suis, a flagellate parasite which is distributed across multiple continents, was initially considered to be solely opportunistic and otherwise nonpathogenic (Tachezy et al., 2002). However, later research suggested that *T. suis* (also known as *Tritrichomonas foetus*) is a facultative pathogen which elicits chronic diarrhea, flatulence, tenesmus, and fecal incontinence in cats (Doi et al., 2012; Lun et al., 2005; Slapeta et al., 2012). Similarly, *T. buttreyi* could be detected in pig fecal samples and was considered a commensal organism (Rivera et al., 2008). However, studies have shown that *T. buttreyi* may infect and cause illness in certain animals. For example, *T. buttreyi* can cause diarrhea in cows (Castella et al., 1997).

P. hominis was found as an opportunistic pathogen causing diarrhea in gastrointestinal tracts in humans, pigs, dogs, monkeys, rats, and cats (Honigberg et al., 1968; Li et al., 2014; Wenrich, 1944). In addition, *P. hominis* was associated with human respiratory tract infections and rheumatoid arthritis (Compaore et al., 2013; Mantini et al., 2009).

Molecular methods are accurate and sensitive in discriminating between strains and species of *Tritrichomonas*. Previous studies have identified the small subunit ribosomal RNA (SSU rRNA) gene as a genetic marker for differentiating species of Trichomonadida (Dufernez et al., 2007; Gookin et al., 2007; Grabensteiner et al., 2010; Reinmann et al., 2012). In the present study, the SSU rRNA gene was used in nested PCR for the identification of *T. suis, T. buttreyi* and *P. hominis* in 158 fecal specimens from healthy pigs from farms in Changchun city, Jilin Province, northeast China. The genetic similarities among *T. suis, T. buttreyi* and *P. hominis* in pigs and other hosts were also analyzed. To the best of our knowledge, this is the first report of *T. suis, T. buttreyi* and *P. hominis* infection in Chinese pigs.

2. Materials and methods

2.1. Fecal specimen collections

Fresh fecal samples were collected from 158 pigs from 3 farms which were located in different suburban regions of Changchun city; 62 samples were from the first-, 49 samples from the second-, and 47 samples from the third farm. Samples were collected

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Table 1
Primers and PCR conditions.

Primers	PCR conditions
First PCR for *T.suis*, *T. buttreyi*, and *P. hominis*	
FF: 5′ -GCGCCTGAGAGATAGCGACTA- 3′ RR: 5′ -GGACCTGTTATTGCTACCCTCTTC- 3′	95 °C for 10 min, then 30 cycles (95 °C for 60 s, 60 °C for 60 s, and 72 °C for 60 s), followed by 72 °C for 10 min
Second PCR for *T. suis*	
sF: 5′ -GGTTGTTTGTATAGGATTGC- 3′ sR: 5′ -TGCCCTCATAAAAGGACAA- 3′	95 °C for 10 min, then 30 cycles (95 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s), followed by 72 °C for 10 min
Second PCR for *T. buttreyi*	
bF: 5′ -GTTTTTTCTCAGGCAGCAATG- 3′ bR: 5′ -GCAACCTAGAAACCTAGGCG- 3′	95 °C for 10 min, then 30 cycles (95 °C for 45 s, 60 °C for 45 s, and 72 °C for 45 s), followed by 72 °C for 10 min
Second PCR for *P. hominis*	
hF: 5′ -TGTAAACGATGCCGACAGAG- 3′ hR: 5′ -CAACACTGAAGCCAATGCGAGG- 3′	95 °C for 10 min, then 30 cycles (95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s), followed by 72 °C for 10 min

between October and December of 2012. The pigs were sorted into 3 groups according to age: pre-weaned (<30 days, $n = 32$), weaned pigs (30–60 days, $n = 71$) and growing pigs (>60 days, $n = 55$). All samples were frozen and stored at −20 °C until DNA extraction.

The present investigation was carried out in accordance with strict guidelines/recommendations from the Guide for the Animal Care and Welfare Committee of Jilin University.

2.2. DNA extraction and PCR analysis

Genomic DNA was extracted from a 200 mg sample of each fecal sample using the Stool DNA Rapid Extraction Kit (TIANGEN, China) following manufacturer instructions. DNA was eluted in a volume of 200 μl. For PCR primer design, SSU rRNA sequences from trichomonads were first retrieved and aligned using the ClustalX 1.81 package, then conserved stretches of DNA were identified for primer design using Primer Premier 5 Design Program. The primer sequences and nested PCR conditions used in this study are shown in Table 1. All the first PCR reaction mixtures contained 2 μl 10 × Ex Taq reaction Buffer (Mg^{2+} plus), 2 μl dNTP mixture (2.5 μM), 0.5 μl each of the respective primer set (20 μM), 1 U Ex Taq (TaKaRa, Japan), and 2 μl of extracted DNA. The total reaction volume was adjusted to 20 μl with distilled water. All the second PCR reactions contained 5 μl 10 × Ex Taq Buffer ($Mg2^{+}$ plus), 5 μl dNTP mixture (2.5 μM), 1 μl each of the respective primer set (20 μM), 2.5 U Ex Taq (TaKaRa, Japan), and 2 μl of the first PCR product. The total reaction volume was adjusted to 50 μl with distilled water. All PCR assays were performed in duplicate. DNA was replaced by water for a negative control.

2.3. Sequence and phylogenetic analysis

PCR products were analyzed by electrophoresis in a 1% agarose gel and visualized with ethidium bromide staining. The target PCR amplification products were purified using a Biospin Gel Extraction Kit (BIOER, China). The purified DNA fragments were cloned into pMD18-T vector (Takara, Japan) and sequenced in both directions. Sequence reads were analyzed using Chromas Pro 1.33 (Technelysium Pty Ltd., Helensvale, Queensland, Australia), after which nucleotide sequences of the three species were analyzed and aligned with *Tritrichomonas* reference sequences (Fig. 1) using ClustalX 1.81 package. Neighbor-joining trees were constructed using MEGA 4 and the evolutionary distances calculated by Kimura 2-parameter model. The reliability of cluster formation was evaluated by bootstrapping with 1000 replicates.

2.4. Assessment of sensitivity

Uninfected fecal samples (200 mg) were spiked with 10 μl aliquots of ten-fold serially diluted *P. hominis*, *T. suis*, or *T.buttreyi* (stored in our laboratory), ranging from 1 to 1000 organisms which were determined with a hemocytometer. A volume of 200 μl DNA was finally obtained by DNA extraction as described above. Nested PCRs (2 μl DNA /reaction) were performed as described above. Two μl DNA was equivalent to 0.01, 0.1, 1, and 10 organisms. DNA was replaced by water for a negative control. Nested PCR products were then analyzed by electrophoresis in a 1% agarose gel.

2.5. Statistical analysis

Comparisons of infection rates of different age groups of *T. suis, T. buttreyi* and *P. hominis* were performed by Chi-square testing using SPSS 18.0, p-value <0.05 was considered significant.

3. Results

3.1. *Frequency of* T. suis, T. buttreyi *and* P. hominis *in pigs*

Among the 158 pig specimens analyzed by nested PCR, 68 pigs (43.04%) were positive for one or two known members of the order Trichomonadida.

Nine samples (12.03%) tested positive for *T. suis*, with 12.50% of pre-weaned, 16.90% of weaned, and 5.45% of growing pigs. The difference in infection rate for *T. suis* was not significant between any two groups. Twenty-three samples (14.57%) were positive for *T. buttreyi* including 12.50% of pre-weaned, 11.27% of weaned, and 20.00% of growing pigs. The difference in infection rate for *T. buttreyi* was not significant between any two groups. Thirty-eight samples tested positive for *P. hominis* (24.05%), with 31.25% of pre-weaned, 21.13% of weaned, and 23.64% of growing pigs. The difference in infection rate for *P. hominis* was not significant between any two groups.

One sample from the growing pig group tested positive for both T. *suis* and *T.buttreyi*. Four pigs (one weaned pig and three growing pigs) tested positive for both *T. suis* and *P. hominis*. Nine pigs (one pre-weaned pig, two weaned pigs, and six growing pigs) tested positive for both *T. buttreyi* and *P. hominis*. No samples tested positive for all three parasites.

3.2. Gene sequencing analysis

Nested PCR resulted in specific bands of approximately 452 bp (*T. suis*), 623 bp (*T. buttreyi*), and 339 bp (*P. hominis*). All specific bands were recovered, sequenced, and submitted to GenBank (Accession numbers: KM205209, KM205210, KM205211, KM205212, and KM205213). Neighbor-joining trees were constructed to assess phylogenetic relationships between trichomonad species, and clearly showed our sequences obtained from fecal

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