PCR-based Discrimination of *Toxoplasma gondii* from Pigs at an Abattoir in Okinawa, Japan

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ABSTRACT. To determine the prevalence of the 3 primary clonal lineages of *Toxoplasma gondii* (strain types I, II, and III) in pigs in Okinawa Prefecture, we analyzed lymph node samples that had been collected at an abattoir by PCR analysis using primers specific for the *Toxoplasma gondii* *SAG2* locus. This study revealed the presence of this parasite in 57 out of 101 samples examined. Restriction fragment length polymorphism (RFLP) in PCR-amplified *SAG2* products was used to group strains into one of the three genotypes of *T. gondii*. Genotypes I and II were equally predominant, accounting for 22 (44.9%) and 23 (46.9%) of 49 *SAG2*-positive samples, respectively, while the type III strain was found in only 4 (8.2%) of the 49 samples. The other 8 samples were indistinguishable by PCR-RFLP analysis. Polymorphisms for the 3 genotypes were confirmed at the sequence level for several samples using the sequences from the RH strain, the Beverley strain, and the C56 strain as references. On the other hand, the dihydropteroate synthase gene, which is responsible for sulfonamide resistance, was amplified in 40 of 54 *SAG2*-positive samples by PCR with the specific primers, and further RFLP and sequence analysis revealed that none of them carried the drug-resistant form of the *dhps* gene. This is the first report of genotyping of *T. gondii* distributed in Japan.

KEY WORDS: genotype, swine, *Toxoplasma gondii*.

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The protozoan *Toxoplasma gondii* is an obligate intracellular parasite that infects a wide range of warm-blooded vertebrates. Between 15 and 85% of the world adult human population is chronically infected with *T. gondii*, depending on geographical location [7]. Although usually benign in immunocompetent individuals, *T. gondii* infection presents a significant health risk in the developing fetus [5] and in immunocompromised patients. Oral ingestion of tissue cysts in undercooked meats and infectious oocytes from the environment are two primary routes of *T. gondii* infection [7], and pigs are considered one of the major sources of meat responsible for oral transmission to humans [6]. The number of cases of toxoplasmosis in pigs in Japan has dramatically decreased. While the abandonment of slaughtered pigs as a result of toxoplasmosis at abattoirs in this country has decreased to almost 1/7 in the last twenty years, the number of cases is certainly not small in Okinawa prefecture, with 46 (of the domestic total) cases in 2003 (http://www.dbtk.mhlw.go.jp/toukei/kouhyo/indexkk_24_1.html).

The genome of *T. gondii* appears to be considerably conserved, indicating the clonal population structure of this geographically widespread parasite [11]. In fact, the vast majority of *T. gondii* isolates studied until now belong to only 2 or 3 clonal lineages, designated as types I, II, and III, which occur in animals and humans [1, 11]. From a technical point of view, many tools have been successively used for analyzing the genetic diversity of *T. gondii* as well as that of other microorganisms. The PCR-restriction fragment length polymorphism (RFLP) method on single-copy genes is the most commonly used method for typing *T. gondii* isolates [4]. Nested PCR-RFLP analysis at the polymor-

phic surface antigen 2 locus (*SAG2*) described by Howe *et al.* [12] was highly sensitive and also provided a rapid unambiguous assignment of the *T. gondii* genotype. On the other hand, there have been few reports regarding the genotypes of the parasite distributed in Japan. The objective of this study was to determine the lineage types of *T. gondii* associated with *T. gondii* infection in pigs in Okinawa, Japan. For this purpose, genetic analysis of the *SAG2* locus by PCR analysis of the DNA obtained directly from tissue samples containing the parasite was performed, omitting the preceding process of isolation in mice or cell cultures. In addition, we examined whether any of the parasites detected in collected samples are sulfonamide-resistant by analyzing the dihydropteroate synthase (*dhps*) gene of *T. gondii*. Aspinall *et al.* [2] described a single amino acid polymorphism with *dhps* (at codon 407), which was causally associated with sulfonamide drug resistance.

A total of 101 lymph node (mainly mesenteric) samples with hemorrhagic or necrotic lesions suspected of toxoplasmosis were obtained from 91 pigs at an abattoir on Okinawa Island from 2003 through 2005. The collected specimens were preserved at –20°C until use after the presence of *T. gondii* tachyzoite had been determined by microscopic examination of an acridine orange-stained stamp-smear sample in every case. Fifty-four of 101 lymph node samples were microscopically positive. Parasite DNA was extracted from the lymph node samples using the SepaGene kit (Vio-gene, U.S.A.) according to the manufacturer's protocol.

Genotype analysis of *T. gondii* was performed by RFLP of the amplified *SAG2* gene using two nested PCR primer sets, each amplifying the 5' and 3' ends of the gene [10].

PCR amplification was performed with 1 μ l of DNA template in 50 μ l of a reaction mixture containing: 10 mM Tris-HCl, pH8.3; 50 mM KCl; 2 mM MgCl₂; 200 μ M dNTPs; 0.5 μ M each of oligonucleotide primers; and 1.25 units of *Taq* DNA polymerase (Takara, Japan). Nested PCR was performed with 50 pmol of primers and 1 μ l of the first PCR-product diluted to 1/10 in water as the template. All PCRs were performed in an automatic DNA thermal cycler (Model TP600 Takara). The first step of amplification was 5 min of denaturation at 94°C. This step was followed by 40 cycles, with 1 cycle consisting of 45 s at 94°C, 45 s at the annealing temperature for each pair of primers, and 60 s at 72°C. The final cycle was followed by an extension step of 10 min at 72°C. The 5' end was amplified using primers SAG2F4 (5'-GACCTCGAACAGGAACAC-3') and SAG2R4 (5'-GCATCAACAGTCTTCGTTGC-3') in the first amplification at an annealing temperature of 60°C. In the second reaction, the internal primers SAG2F (5'-GAAATGTTTCAGGTTGCTGC-3') and SAG2R2 (5'-GCAAGAGCGAACTTGAACAC-3') were used, with 58°C as the annealing temperature. Amplification of the 3' end was performed with primers SAG2F3 (5'-TCTGT-TCTCCGAAGTGACTCC-3') and SAG2R3 (5'-TCAAAGCGTGCAATTATCGC-3') for the first amplification at an annealing temperature of 58°C and with the internal primers SAG2F2 (5'-ATTCTCATGCCTCCGCTTC-3') and SAG2R (5'-AACGTTTCACGAAGGCACAC-3') for the second amplification at an annealing temperature of 55°C. The 5'-end amplification is expected to yield a product of 241 bp, while the 3'-end amplification must give a product of 221 bp in both cases for any strain of *T. gondii*. After amplification, the PCR products were digested with *Sau*3AI (5'-end products) and *Hha*I (3'-end products). The PCR products and the restriction fragments were analyzed by 1.2–1.5% agarose gel electrophoresis. Concerning the representative sample of each *SAG2* type, the complete nucleotide sequences of the partial *SAG2* were determined from both strands using the ABI PRISM 3100 or 3700 Genetic Analyzer (Applied Biosystems, U.S.A.) with a big dye chain terminator kit (Applied Biosystems) in order to confirm the *SAG2* type identified by restriction analysis. The nucleotide sequences were also applied to a Basic Local Alignment Search Tool (BLAST) on the DNA Data Bank of Japan (DDBJ) database for homology analysis against other *SAG2*'s of reference *T. gondii* strains.

All *SAG2*-positive samples were screened for their mutation in *dhps* at codon 407. This was achieved by nested PCR amplification of the *dhps* gene fragment encompassing codon 407 [3] followed by RFLP and sequencing. The PCR primer pair FOOD1 (5'-GGAACATCCGCTGAAGCT-CATGG-3') and FOOD2 (5'-CAGAGAATCCAGT-TGTTTCGAGG-3') was used in the initial PCR reaction, and the amplification reaction was performed over 35 cycles under standard conditions (detailed above) at an annealing temperature of 57°C. The resulting amplification products were diluted to 1/10 in water, and a second amplification was performed with the internal primers FOOF3 (5'-CAGTCCAGACTCGTTCACCGATC-3') and FOOD4 (5'-CCGGAATAGTGTATACTTGTAG-3') using 1 μ l of the diluted product as the template under the same reaction conditions. All of the final PCR products were digested with *Cfr*13I, and six of 40 fragments amplified were directly sequenced (as detailed above for *dhps*).

Direct amplification of both ends of the *SAG2* gene was successful in 49 of 101 (48.5%) lymph node samples with toxoplasmosis-like lesions. In another five cases (8.8%), only the 5' end of the *SAG2* gene was amplified, and, in another three (5.3%), only the 3' end was amplified (Table 1). In order to determine the *SAG2* type of *T. gondii* in the samples, the *SAG2* PCR products were digested with restriction enzymes, and their type was determined according to the restriction pattern after separation by agarose gel electrophoresis.

Figure 1 shows the results of a representative experiment. Digestion of the 5' amplification products with *Sau*3AI distinguished allele 3 (genotype III) from alleles 1 and 2 (genotypes I and II), and digestion of the 3' amplification products with *Hha*I distinguished allele 2 (genotype II) from alleles 1 and 3 (types I and III) (Fig. 1A, B). Genotyping of the 49 samples with complete characterization rendered the following results: 22 of type I (44.9%), 23 of type II (46.9%), and 5 of type III (8.2%). Eight strains that were partially characterized resulted in type I or II (non-type III) or type I or III (non-type II) (Table 1). It was previously reported that either the 5' or the 3' end of *SAG2* was amplified in some cases, when clinical samples of human toxoplasmosis were directly analyzed [6]. No mixed infections were detected. Interestingly, the *SAG2* genotype of *T. gondii* from pigs varied with the shipment farm. However, more than two different genotypes were detected in pigs

Table 1. Genotypes of *T. gondii* found in lymph node samples of pork pigs in Okinawa

No. of samples (%)	Nested PCR-RFLP results ^{a)}			Reference strains
	<i>SAG2</i> -5'	<i>SAG2</i> -3'	Type	
22 (38.6)	–	–	I	RH
23 (40.4)	–	+	II	Beverley
4 (7.0)	+	–	III	C56
3 (5.3)	NA	–	non-II	
5 (8.8)	–	NA	non-III	

a) NA means not amplified. (+) and (–) mean cleaved and not cleaved with the restriction enzymes, respectively.

Fig. 1. Representative result of nested PCR-RFLP analysis of the *SAG2* from *T. gondii*-infected samples. (A) *Sau3AI* restriction analysis of the 5' amplification products and (B) *HhaI* restriction analysis of the 3' amplification products. Lanes I, II, and III are the isolates that were characterized as genotypes I, II, and III in this study, respectively. Lane M is the molecular-weight marker.

Fig. 2. Nested PCR-RFLP analysis of the *dhps* gene. (A) *Cfr13I* digestion of *dhps* PCR-amplified DNA from several *T. gondii* parasites with each *SAG2* genotype. (B) Restriction patterns presumed by the nucleotide sequences of *dhps* from the database of reference strains and sulfonamide-resistant mutant with mutation at codon 407. Lane 1 shows the PCR products of the *dhps* gene. Lanes 2–5, Lanes 6–10, and Lanes 11–12 are the restriction fragments of the *dhps* genes from strain types I, II, and III, respectively. W is the wild type of the *dhps* gene. Mu is a sulfonamide-resistant mutant. Lane M is the molecular-weight marker.

Table 2. Sequence analysis of the *T. gondii dhps* gene and nucleotide mutation positions

Location ^{a)}	Sequence of the <i>dhps</i> gene (n)			Sulfonamide resistance ^{b)}
	Type I (2)	Type II (2)	Type III (2)	
Exon 1 (codon 407)	AAC	AAC	AAC	wild type
Intron 1 (nt 1513)	AGA	AAA	AAA	–
Intron 1 (nt 1565)	TCT	TTT	TTT	–

a) nt, nucleotide. b) Aspinall *et al.* [2] reported that the *dhps* mutation at codon 407 within exon 1 associated with the sulfo-resistant strain of *T. gondii* changed from asparagine (AAC) in the sensitive wild type to aspartate (GAC).

from a farmer who had farms at different locations (data not shown).

Fourteen PCR products of the 5' and 3' end of the *SAG2* gene from seven samples with each genotype were directly sequenced. The nucleotide sequences for the samples (parasites) that were characterized as genotypes I, II, and III in this study were all completely identical with those of their reference strains, the RH strain (M33572, type I, virulent for mice, human origin), the Beverley strain (AF249697, type II, avirulent for mice and cyst-forming, rabbit origin), and the C56 strain (AF249698, type III, intermediately virulent for mice, chicken origin) (Table 1).

It is known that there is a bias in the frequency of *T. gondii* strain types isolated from human toxoplasmosis and animal infections. The type II strains are associated with the

majority of human toxoplasmosis cases [10–12]. Similarly, type II strains were prevalent in pigs. It was reported that 83.7% of the isolates were type II strains when the isolates of *T. gondii* collected from pigs at an abattoir in Iowa were examined [13]. On the other hand, it was reported in Brazil that *T. gondii* isolates from chickens were types I and III and that no type II strains had been found [8]. Most of the infected commercial meat samples carried *SAG2* type I parasites in the UK [3]. The prevalence of *T. gondii* strain types observed in this study differs from that reported previously. This difference might be explained by the selection and isolation of the parasite in the process of culture before PCR analysis. Alternatively, it might be explained by geographic property.

All the samples shown to be *Toxoplasma*-positive by

SAG2 amplification were then further tested with nested PCR analysis using primers specific for the *T. gondii dhps* gene. The amplified region encoding amino acid residue 407 was previously described as critical for sulfonamide resistance in *T. gondii* [2]. The amplification of the *dhps* gene fragment was successful in 40 of 57 *SAG2*-positive samples. RFLP analysis with the *Cfr13I* and DNA sequence analysis of these *dhps* fragments revealed that all samples were similar to the wild-type *dhps* in respect to amino acid residue 407 (Fig. 2, Table 2). Additional sequence analysis for 6 samples identified two nucleotide mutations located at nucleotide 1513 and 1565 within intron 1 of the *dhps* gene. These polymorphisms distinguished genotype I from genotypes II and III (Table 2). There are still several cases of *T. gondii* infection in pigs in Okinawa, though the disease is very rare in other regions of Japan. The acquisition of tolerance to sulfonamide drugs by the parasite was thus anticipated; however, such a probability seemed to be low. The present study may provide useful information regarding not only the epidemiology of toxoplasmosis in pigs but also distribution of the infection source of human toxoplasmosis. Further genetic analysis using a more polymorphic marker and multilocus genotyping might be necessary to reveal atypical or recombinant genotypes, which might exist in the study area.

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