Functional characterization of *Toxoplasma gondii* SAG1-related sequence 2 and evaluation of its potential in serodiagnosis

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トキソプラズマの SRS2 分子の機能解析と血清診断抗原としての評価

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Contents

Abbreviations and unit abbreviations

B	BCA	Bicinchoninic acid

	BSA	Bovine serum albumin
H	HRP	Horseradish peroxidase
C	cDNA	Complementary deoxyribonucleic acid
	HXGPRT	Hypoxanthine xanthine guanosine phosphoribosyltransferase
	CI	Cytopathic effect
I	CRISPR/CAS9	Clustered Regularly Interspaced Short Palindromic Repeats- associated protein
	IFAT	Immunofluorescent antibody test
	IHA	Indirect hemagglutination assay test
D	DAPI	4',6-diamidino-2-phenylindole
	DHFR	Dihydrofolate reductase
	DKO	Double knockout
	DNA	Deoxyribonucleic acid
E	*E. coli*	*Escherichia coli*
	EDTA	Ethylenediaminetetraacetic acid
	ELISA	Enzyme linked immunosorbent assay
F	FBS	Fetal bovine serum
G	GFP	Green fluoresent protein
	GPI	Glycophosphatidyliositol
	GRA	Dense granule antigen
	gRNA	Guide ribonucleic acid
	GST	Glutathione S-transferase
H	HFF	Human foreskin fibroblast
	HIV	Human Immunodeficiency Virus

	IFAT	Immunofluorescent antibody test
	IFN-γ	Interferon gamma
	IgG	Immunoglobulin G
	IHA	Indirect hemagglutination
	IPTG	Isopropyl-β-D-thiogalactopyranoside
	IFA	Indirect fluorescent antibody assay
K	kDa	Kilodalton
L	LAT	Latex agglutination test
M	MAT	Modified agglutination test
	MEME	Eagle's minimum essential medium
N	NLS	Nuclear localization signal
P	PAM	Protospacer-adjacent motif
	PBS	Phosphate buffered saline
	PBST	Phosphate buffered saline with Tween-20
	PCR	Polymerase chain reaction
R	r	Recombinant
	RT	Room temperature
	RNA	Ribonucleic acid
S	SAG	Surface antigen
	SDS	Sodium dodecyl sulfate
	SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
	SM	Skimmed milk
	SPF	Specific pathogen free

	SRS	SAG1-related superfamily
T	Tg	*Toxoplasma gondii*
W	WT	Wild type

Unit abbreviations

bp	Base pair
µl	Microliter
°C	Degree Celsius
µg	Microgram
g	Gram
nm	Nanometer
mg	Milligram
µm	Micromolar
min	Minute
h	Hour
%	Percentage
ml	Milliliter
mM	Millimole

General introduction

1. *Toxoplasma gondii* and toxoplasmosis

Toxoplasma gondii is an obligate intracellular protozoan parasite belonging to the phylum Apicomplexa. *T. gondii* was first found in a mononuclear cell isolated from the liver and spleen of the pedicle rats by the French scholars Charles Nicolle and Louis Manceaux in 1908 in North Africa (Nicolle and Manceaus, 1908). It has a worldwide distribution and a high prevalence in many areas of the world, some scholars estimate that a third of the world's human population is infected with *T. gondii*, up to 95% in local areas, and about 200,000 new cases of toxoplasmosis occur every year (Montoya and Liesenfeld, 2004). There are no clear symptoms of toxoplasmosis in healthy adults. However, toxoplasmosis is life threatening in immunocompromised individuals (such as those with HIV infection or on immunosuppressive therapies or post transplantation) (Montoya et al., 2004; Sensini et al., 2006). In pregnant women, infection with the parasite can cause vertical infection resulting in stillbirth, neonatal malformations or abortion (Tenter et al., 2000; Jones et al., 2003). *T. gondii* is an important zoonotic pathogen that has been studied by many researchers in recent years. However, there are still many mysteries of its epidemiology, pathogenic mechanism and natural life cycle.

2. Life cycle of *T. gondii*

T. gondii can infect a wide range of hosts and has a facultative heteroxenous life cycle due to its ability to survive in multiple hosts (Levine, 1961; Dubey and Beattie, 1988; Dubey, 1998). The intermediate hosts which include almost all warm

blooded animals, can harbor infective tissue cysts. The Felidae, such as domestic cats, are the definitive hosts that pass the infective oocysts in their feces (Dubey, 1986; Dubey and Beattie, 1988; Jackson and Hutchison, 1989; Dubey, 1993; Frenkel, 2000).

Life cycle of *T. gondii* within the host is divided into two phases: asexual and sexual reproduction. Asexual reproduction occurs in intermediate hosts and also occurs in two phases: first, tachyzoites (or endozoites) replicate rapidly by repeated endodyogeny in host cells. The next phase starts from the last generation of the tachyzoites, that transform into tissue cysts in which bradyzoites (or cystozoites) replicate slowly by endodyogeny (Dubey and Beattie, 1988; Jackson and Hutchison, 1989; Evans, 1992; Dubey, 1993; Dubey et al., 1998). Tissue cysts are mainly distributed in the eye, central nervous system and cardiac muscles because of their high affinity for neural and muscular tissues. They are infectious and may persist for the rest of life of the hosts (Dubey, 1998; Dubey et al., 1998).

The sexual stage of the parasite only develops in the definitive hosts. The infective tissue cysts multiply rapidly by repeated endopolygeny in the epithelial cells of the small intestine. The gamogony and oocyst are then formed in the epithelial cells of the small intestine. Unsporulated oocysts are released into the environment with the faeces and later transform into infectious oocysts. Although cats secrete oocysts only in a short period, they release large amounts of oocysts which are resistant to environmental conditions including freezing and drying (Dubey et al., 1970; Dubey and Beattie, 1988; Jackson and Hutchison, 1989; Evans, 1992; Dubey, 1993; Dubey et al., 1998; Bhopale, 2003).

T. gondii has three infectious stages and these include bradyzoites, tachyzoites and sporozoites. Both definite hosts and intermediate hosts can be infected by one of the above infectious stages. Routine infection pathways include foodborne, animal to human, congenital, and organ transplant or blood transfusion transmissions. Foodborne transmission happens through ingestion of raw or undercooked meat that contains encysted bradyzoites; oral ingestion of food which was contaminated by cutting boards and knives or direct contact with contaminated meat. Animal to human transmission is the result of accidental ingestion of fruit and vegetables or drinking water that is contaminated with oocysts. In congenital transmission, women infected with *T. gondii* during pregnancy infect offspring by transplacental transmission of tachyzoites (Tenter et al., 2000; Jones et al., 2012). Organ transplant or blood transfusion transmission happens in people who received organ transplantation from an infected donor. As a result, *T. gondii* may be transmitted between the intermediate hosts, the final hosts or from intermediate to definitive host and vice versa (Tenter et al., 2000, Flegr et al., 2014).

3. SAG1-related superfamily of *T. gondi*

The SAG1-related superfamily (SRS) is one of coccidian-specific multicopy gene families of *T. gondii* (Wasmuth et al., 2009). In total, 182 TgSRS genes have been identified in three *T. gondii* strains using comparative genomic analyses (Wasmuth et al., 2012). A TgSRS gene usually contains one or two domains, every domain consisting of four to six cysteines, which are involved in disulfide bond and a glycophosphatidylinositol (GPI) anchor to attach the cell surface (Manger et al., 1998). TgSRS proteins are the differential expression of numerous closely related

GPI-anchored surface proteins. They are expressed in a unique developmentally modulated manner as different, largely non-overlapping sets of TgSRS antigens (Jung, 2004; Kim, 2007).

These TgSRS proteins perform distinctly different function in the parasite. TgSRS29B (formerly TgSAG1) can elicit a lethal inflammatory ileitis (Rachinel et al., 2004). TgSRS57 (TgSAG3), as a ligand, can mediate the identification and attachment between host cells and parasites (Dzierszinski et al., 2000). TgSRS16B (TgSRS9) plays an important role in transmissible latent infections and reactivation in the intestine (Kim et al., 2007). TgSRS29B and TgSRS34A (SAG2A) have high expression and high immunogenicity which is thought to attract host immunity to facilitate establishment of a chronic infection of the parasite (Lekutis et al., 2001). *T. gondii* expresses and regulates TgSRS super-family to successfully establish chronic infection in the intermediate hosts (Wasmuth et al., 2009).

4. Diagnosis of toxoplasmosis

Development of accurate diagnostic methods is crucial for proper management and control of *T. gondii* infection in humans and animals. A number of diagnostic methods have been developed and widely practiced. They have variable specificities and sensitivities depending on equipment, trained personnel, direct or indirect detection. They include histologic demonstration, isolation of the organism, amplification of specific nucleic acid sequences and serological tests. These methods can be used singly or in combination (Montoya, 2002). Clinical signs of toxoplasmosis are non-specific and are similar to several other infectious diseases resulting into unreliability on a definite diagnosis (Hill and Dubey, 2002).

Serological diagnosis is commonly used to detect the antibodies against *T. gondii*. A number of serological procedures are available for detection of *T. gondii* antibodies, including Sabin-Feldman test, direct agglutination test, indirect fluorescent antibody assay (IFA), immunosorbent agglutination assay test (IAAT), indirect hemagglutination assay, latex agglutination test (LAT) and enzyme-linked immunosorbent assay (ELISA) (Frenkel et al., 1970; Remington et al., 1995; Hill and Dubey, 2002). LAT is considered the "gold standard" for detecting *T. gondii* infection, but has low specificity and a high cost (Dannemann, 1990). ELISAs seem to be the most suitable for routine mass diagnosis such as in sero-epidemiological investigations (Montoya, 2002). The specificity and sensitivity of these serologic methods depend mostly on the diagnostic antigen used. Several recombinant proteins hold potential to be used for diagnosis in cats and humans, and among them are the major surface antigens (TgSAGs) and dense granule antigens (TgGRAs) that have been shown to be good candidates (Kotresha and Noordin, 2010).

5. Aims of the present study

T. gondii is an important zoonotic parasite not only having a serious public health concern in humans, but also a significant reproductive and economic impact in animals. Thus development of accurate diagnostic methods is crucial for proper management and control of *T. gondii* infection in humans and animals.

A better understanding of the antigens that play important roles for growth or virulence of *T. gondii* is important for developing a sensitive and specific diagnostic method, even in designing a safe and effective vaccine. The TgSRS superfamily

express largely nonoverlapping sets of TgSRS antigens which are thought to dominate the surface of *T. gondii* (Kim, 2007). The TgSRS antigens might provide some types of protective barrier, act as diverse arrays of cellular receptors, and/or function as immune activators or mediators of immune evasion that facilitates parasite survival and propagation. SAG1-related sequence 2 (TgSRS2) is a major tachyzoite surface antigen and has been identified to be expressed on the surface of *Toxoplasma* tachyzoites (Manger, 1998). The survival rate of mice significantly increased when infected with lethal dose of TgSRS2 overexpressing mutant parasites (Wasmuth et al., 2012). These indicate TgSRS2 may be an important virulence factor in *T. gondii*.

The domestic cat as a pet and definitive host of the parasite plays an important role in the transmission of *T. gondii*. It may be the main source of contamination, because millions of oocysts may be excreted by domestic cat and can be spread widely to contaminate the environment. Therefore, it is urgent to develop a sensitive and specific diagnostic method for *T. gondii* in cats. In recent years, a growing number of surveys of *T. gondii* infection in sheep have been carried out worldwide, and several surveys have shown that *T. gondii* infection in sheep is common in some provinces of China. However, there is a gap in knowledge of *T. gondii* infection in sheep in northern China.

The aims of current study can be summarized as follows: (1) to preferably understand the function of TgSRS2 in *T. gondii*; (2) to develop an accurate diagnostic method of *T. gondii* infection in cats; (3) to comprehensively understand the occurrence of toxoplasmosis in sheep in northern China.

Chapter 1

Functional analysis of *Toxoplasma gondii* SAG1-related sequence 2 using CRISPR/CAS9

1-1. Introduction

Toxoplasma gondii is one of the most successful protozoan parasites because of its ability to infect any warm-blooded animal (Robert-Gangneux, 2012). The other coccidian parasites including *Plasmodium*, *Theileria* and *Cryptosporidum*, however, possess a limited host range. Their patterns of infection depend on the physical interactions with the host. Some apicomplexans have evolved surface proteins that promote the parasite's attachment, invasion and evasion of host immunity (Morrissette, 2002; Templeton, 2007; Wasmuth et al., 2012).

The SAG1-related superfamily (SRS) is one of the apicomplexan-specific multicopy gene families in the *T. gondii* genome. Unlike *P. falciparum var* genes, that only express a single locus by increasing ectopic recombination rate to promote rapid sequence variation, the TgSRS proteins are present in a developmentally regulated manner which show different and largely non-overlapping set of proteins (Scherf et al., 1998; Jung, 2004; Wasmuth et al., 2009; Wasmuth et al., 2012). These TgSRS proteins perform distinctly different functions in the parasite. TgSRS29B (SAG1) is a virulence determinant that can elicit a lethal inflammatory ileitis in mice and mediates

adhesins (Mineo et al., 1993; Rachinel et al., 2004). TgSRS57 (TgSAG3) plays an important role in recognition and attachment between host cells and parasites (Dzierszinski et al., 2000). TgSRS16B (TgSRS9) plays an important role in transmissible latent infections and reactivation in the intestine (Kim et al., 2007). TgSRS29B and TgSRS34A (TgSAG2A) have high expression and high immunogenicity which is thought to attract host immunity to facilitate establishment of a chronic infection of the parasite (Lekutis et al., 2001). *T. gondii* may express and regulate TgSRS super-family to successfully establish chronic infections in the intermediate host (Wasmuth et al., 2009).

T. gondii expresses high level of SAG1 and SAG2A proteins to induce strong immunity during infection (Boothroyd et al., 1998). The double knockout (DKO) TgSAG1 and TgSAG2A mutant showed apparent upregulation of TgSRS2 protein, with expression level similar with the mouse avirulent type II and III parasites (Wasmuth et al., 2012). Upregulation was more likely to be regulated posttranscriptionally. Importantly, the virulence of DKO mutant was significantly attenuated. Subsequently, researchers constructed TgSRS2 overexpression transgenic parasites based on type I RH strain, and also observed that the mutant parasites attenuated the virulence of the wild type in mice by 60 to 70％ (Wasmuth et al., 2012). These date indicate the TgSRS2 proteins play an important role in the virulence of *T. gondii*.

1-2. Materials and methods

Ethics statement

The recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology, Japan were strictly followed in this study. The protocol was approved by the Committee on the Ethics of Animal Experiments at the Obihiro University of Agriculture and Veterinary Medicine. All painful experimental treatments were implemented after general anesthesia induced with isoflurane.

Animals

Six-week-old female ICR and BALB/c mice, and female Japanese white rabbits were purchased from Clea Japan. They were fed in a specific animal facility at the National Research Center for Protozoan Diseases of Obihiro University of Agriculture and Veterinary Medicine.

Cell and parasite cultures

In the current study, *T. gondii* virulent type I RH with HXGPRT deficient and avirulent type II PLK strains were used. The parasites were maintained in Vero cells that were cultured in EMEM (Sigma) supplemented with 8% FBS (Biowest) and 1% penicillin-streptomycin (Sigma) at 37 °C and 5% CO_2. To purify the tachyzoites, host cells infected with parasites were washed with PBS and removed from flask with a cell scraper (BD Biosciences Inc.). The final pellet was re-suspended in EMEM, passed through a 27-gauge needle three times, and filtered through a 5.0-μm pore filter (Millipore).

Amplification and cloning of the TgSRS2 gene

Total RNA of *T. gondii* was extracted using Trizol (Life Technologies) from 5×10^7 parasites and then reverse transcribed to cDNA using SuperScript III First Strand Synthesis Kit (Invitrogen, USA) according to the manufacturer's protocol. To

compare the nucleotide sequences of TgSRS2 from RH and PLK strains, a pair of primers was designed based on the predicted TgSRS2 sequences (*Toxoplasma* Genomics Resource TGGT1_233480 and TGME49_233480). The TgSRS2 open reading frame full-length was amplified from cDNA of the RH and PLK strains using the above described primers with *Sac*I and *Not*I restriction enzyme sites (underlining): forward 5'-CGAGCTCATGGCGACGCGTGCGTCTT-3' and reverse 5'-ATAAGAATGCGGCCGCATCAATAGGCAAGTGCCGTC-3'. The PCR products were cloned into the pGEM-T Easy vector (Promega, USA). The positive plasmids pGEM-T-TgSRS2 were sequenced by a Big Dye Terminator Cycle Sequencing kit (Applied Biosystems) and an ABI PRISM 3100 genetic analyzer (Applied Biosystems).

Expression of TgSRS2 recombinant protein

The cDNA fragment of TgSRS2 from PLK strain was obtained from pGEM-T-TgSRS2 plasmid by cutting *Sac*I and *Not*I restriction sites. The fragment was inserted into *Escherichia coli* expression vector pGEX-4T-1 (Amersham Pharmacia Biotech, Sweden). Recombinant protein was expressed in *E. coli* BL 21 DE3 cells. The recombinant TgSRS2 with GST tag (rTgSRS2) was expressed in *E. coli* BL 21 DE3 cells at 37 °C under induction with 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Wako, Japan). The purification of rTgSRS2 was performed as described previously (Fereig et al., 2017). In addition, the recombinant TgSAG1 fused with GST tag (rTgSAG1) (Kimbita et al., 2001) was also purified for preparation of anti-rTgSAG1 sera.

Production of polyclonal antibodies against TgSRS2 and TgSAG1

Seven-week-old female ICR mice were intraperitoneally immunized with 100

μg of purified rTgSRS2-GST emulsified in an equal volume of Freund's complete adjuvant (Sigma, USA) on day 0. Mice were immunized again with 50 μg of the same protein in Freund's incomplete adjuvant (Sigma, USA) on day 14 and 28 after the first immunization. The anti-rTgSRS2 sera were collected on day 14 after the last immunization. Mice anti-rTgSAG1 sera were similarly prepared. In the same period, a female Japanese white rabbit was subcutaneously injected with 1 mg of purified rTgSAG1-GST to produce rabbit anti-rTgSAG1 sera.

Western blot analysis

Western blot analysis was performed as previously described (Fereig et al., 2017). The target sample was mixed with 5×SDS gel loading buffer and heated at 95 °C for 5 min. The proteins were separated on a 12% polyacrylamide gel and transferred to a nitrocellulose membrane (Whatman GmbH, Germany) after SDS. The membrane was blocked with PBS containing 3% (w/v) skimmed milk (PBS-SM) for 1 h at room temperature (RT). After washing twice with PBS-T, the membrane was incubated with mouse anti-TgSRS2 or anti-TgSAG1 serum (1:500) diluted in PBS-SM at room temperature for 1 h. After four washes, the membrane was incubated with HRP-conjugated immunoglobulin G (1:2,000) (Amersham Pharmacia Biotech, USA) for 1 h at RT. After washing five times, the target protein was visualized by VersaDoc^{TM} imaging system (Nippon Bio-Rad Laboratories, Japan) using ECL^{TM} western blotting detection reagents(GE Healthcare, UK).

Indirect fluorescent antibody test (IFAT)

IFAT was carried out as described previously (Leesombun et al., 2016). Briefly, 1×10^5 Vero cells per well in 12-well plates were incubated for 24 h, and then infected with *T. gondii*. They were fixed with 4% paraformaldehyd, 24 h post

infection, and then permeabilized with 0.3% Triton X-100 in PBS for six min at RT. This was followed by washing in PBS and blocking with 3% bovine serum albumin (BSA) in PBS for 30 min at RT. Incubation with mouse anti-SRS2 serum (1:500) in 3% BSA, followed, for 1 h at RT. After washing, the cells were incubated with Alexa 488-conjugated goat anti-mouse fluorescent antibody (1:1000) (Thermo Fisher, USA). Nuclei were labeled with DAPI (Sigma, USA). Finally, the coverslips were fixed on a glass slide and examined by ALL-in-One Fluorescence Microscope (BZ-9000, Keyence, Japan).

Generation of RH and PLK SRS2-knockout parasites

To investigate the function of SRS2 in *T. gondii*, the plasmids (named pHX and pDF) were used to generate SRS2-knockout parasites. Briefly, both the vector backbones contained targeted gene's guide RNA sequence (gRNA), CAS9, GFP, and HXGPRT/dihydrofolate reductase (DHFR) expression cassettes as shown in Fig. 2. In the current study, only the gRNA sequence was changed into TgSRS2 targeting sequence designed using E-CRISP (E-Crisp.org), and the final plasmids were designed as the pHX-RH-SRS2 and pDF-PLK-SRS2. Purified RH and PLK tachyzoites were transfected with 10 μg circular pHX-RH-SRS2 and pDF-PLK-SRS2 DNA respectively using Amaxa Human T Cell Nucleofector Kit (LONZA, Germany), and immediately used to infect Vero cells. Selection of parasites stably transfected resistant to mycophenoloic acid/xanthine or pyrimethamine was performed as described earlier (Donald and Roos, 1998; Reynolds et al., 2001). To isolate clones, these stable transfected parasites were diluted into Vero cells grown on 96-well plates. Single-plaque wells were collected and further analyzed by Western blotting and IFAT.

Invasion assay

Vero cells were cultured at 1×10^5 cells/well in 12-well plates and incubated for 24 h. These cells were then infected with wild types and derivatives of *T. gondii* (three separate wells per experiment). After 2 h post infection, the extracellular parasites were washed away with PBS and MEME supplemented with 8% FBS was added. To determine parasites invasive ability, the infection rates were calculated by IFAT at 24 h post-infection as follows: [(number of SAG1-positive Vero cells) / (200 randomly selected Vero cells)] × 100. Three independent experiments were conducted.

Replication assay

To compare the growth of knockout and wild types parasites, replication assay was performed. RH, PLK, and knockout parasites were purified and used for inoculation of Vero cells in 12-well culture plates (2×10^5 tachyzoites/well). After 2 h of incubation, the extracellular parasites were washed away with PBS, and the parasites were incubated for another 22 h. The percentage of vacuoles containing different number of parasites was counted by IFAT in at least 100 vacuoles per well (three separate wells per experiment). Three independent experiments were conducted.

Fatality assay

Seven-week-old female BALB/c mice were intraperitoneally injected with *T. gondii* (type I, 100 tachyzoites/mouse; type II, 1×10^4 tachyzoites/mouse) (n = 5 for each group). The survival rate and body weight were recorded for 30 days post-infection. Two or three independent experiments were conducted.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 5 software (GraphPad

Software, Inc.) using unpaired Student's *t*-test and two-way analysis of variance (ANOVA) followed by the Tukey-Kramer test. Survival analyses for significant differences were generated using the long-rank method. Results were considered to be statistically significant difference when the *P value* was <0.05.

1-3. Results

Analysis of TgSRS2 amino acid and its native expression

The nucleotide sequence of TgSRS2 amplified from RH and PLK strains were consistent with the predicted sequences of Type I (TGGT1_233480) and Type II (TGME49_233480), respectively. The full length of the open reading frame of TgSRS2 was 1,119 bp and encoded 372 amino acid residues. Amino acid sequence alignment showed that TgSRS2 of PLK contains four amino acid substitution compared to that of RH strain (Fig. 1A). The rTgSRS2 was expressed in *E. coli* as GST-fused protein and its molecular weight was the same as the expected size (Fig. 1B). Furthermore, the purified rTgSRS2 could be recognized by serum from mice experimentally infected with the PLK strain in Western blot analysis (Fig. 1B). The same size of native TgSRS2 was detected in RH and PLK strains by anti-TgSRS2 serum from mice (Fig. 1C).

CRISPR/CAS9-mediated TgSRS2 disruption in RH and PLK strains

Previous studies have shown that CRISPR/CAS9 with specific gRNA can be used to generate target gene disruption in *T. gondii* (Shen et al., 2014; Zheng et al., 2015; Yang et al., 2016; Zheng et al., 2016). To investigate the function of TgSRS2 in *T. gondii*, TgSRS2-knockout parasites (ΔTgSRS2) were generated using CRISPR/CAS9 technology. The knockout plasmids pHX-RH-SRS2 and pDF-PLK-

SRS2 were constructed with same TgSRS2 gRNA sequence (Fig. 2A) and different selectable marker cassettes (Fig. 2B). After transfection and selection, ΔTgSRS2 parasites were identified using western blot (Fig. 2C) and IFAT analyses (Fig. 2D). The expression of TgSRS2 could be detected in RH and PLK strains but not in mutant of each strain. These results confirmed the loss of TgSRS2 in the ΔTgSRS2 parasites.

ΔTgSRS2 parasites exhibit defect in invasive ability *in vitro*

T. gondii possesses the ability to invade and infect any nucleated cell. Previous study indicated that the surface protein TgSAG3, a member of the TgSRS family, was associated with host cell invasion (Dzierszinski et al., 2000). Thus, the role of TgSRS2 in this process was also evaluated in current study. The results of these experiments showed no significant difference in percentage of infected cells between RH and ΔTgSRS2 parasites (Fig. 3A). However, the percentage of infected cells by PLK strain was significantly higher than that infected by ΔTgSRS2-A4 and ΔTgSRS2-C4 parasites (Fig. 3B). The results therefore reveal that TgSR2 plays an important role in host cell invasion in strain-specific pattern.

TgSRS2 is dispensable for *T. gondii* growth *in vitro*

Since the loss of TgSRS2 affected host cell invasion, replication assay was performed to evaluate its effect on parasite growth *in vitro*. After 24 h post infection, the number of parasites per vacuole was counted. The percentages of both RH and PLK strains vacuoles containing specific parasite number were similar to that of their ΔTgSRS2 mutants (Fig. 4A and 4B). These results indicate that TgSRS2 is dispensable for parasite replication in *vitro*.

ΔTgSRS2 strains exhibit attenuated virulence in mice

Previous studies proved that TgSRS2 was an important regulator of acute virulence in mice (Wasmuth et al., 2012). Therefore, I investigated the virulence of ΔTgSRS2 strains in mice. BALB/c mice were intraperitoneally injected with RH (10^2), ΔTgSRS2-B4 (10^2), PLK (10^4, 10^5) and ΔTgSRS2-A4 (10^4, 10^5) tachyzoites. Interestingly, all the mice infected with virulent type I succumbed to the infection within 7-9 days (Fig. 5A), and body weight loss was observed following infection (Fig. 5B). Meanwhile, the survival rates of mice challenged with ΔTgSRS2-A4 (10^4, 10^5) were significantly higher than those challenged with equal number PLK strain tachyzoites (Fig. 5C), and body weight loss following infection was only observed in the mice infected with PLK (10^5) following infection (Fig. 5D). All these results indicate that TgSRS2 is an important virulence factor in PLK.

1-4. Discussion

TgSRS2 was initially identified as a stage-specific member of the SAG1-related gene family that is differentially expressed at the post-transcriptional level in virulent and avirulent strains of *T. gondii* but not at the transcriptional level (Manger et al., 1998; Wasmuth et al., 2012). However, the function of TgSRS2 was unclear. Thus, in the current study, functional analysis of TgSRS2 was performed by knocking out this gene using CRISPR/CAS9 technology. The phenotypic analysis indicates that the ΔTgSRS2 parasites can still invade the host cells and replicate *in vitro*, indicating that TgSRS2 protein is not essential for the development of *T. gondii*. Interestingly, unlike the RH strain, the loss of TgSRS2 in PLK strain significantly affects the invasive ability *in vitro* and virulence in mice. However, no difference was observed in growth of cell lines between the WT and TgSRS2 deficient parasites. The amino acid sequence alignment of the two genotypes reveals that the PLK SRS2 allele possesses

four amino acid polymorphisms relative to the RH allele. Previous study has proved that small amount of TgSRS2 is expressed in the highly virulent type I, whereas high quantity of this protein is detected in the avirulent type II (Manger et al., 1998). In another study, either overexpression caused by double knockout of SAG1 and SAG2A or transoverexpression of type I SRS2 or type II SRS2 in RH strain, dramatically decreased its virulence in mice (Wasmuth et al., 2012). All theses data indicate that the expression level of TgSRS2 is important for its function in *T. gondii*. Thus the different effects in invasion and virulence caused by deletion of TgSRS2 in RH and PLK strains may be related to the difference in its abundance.

T. gondii is able to invade almost any nucleated cells within its mammalian hosts but the mechanism of invasion is not yet fully understood. TgSRS proteins are thought to play an important role in attachment to the host cell. TgSRS2, just like other surface proteins has an N-terminal signal peptide and is anchored with the glycosylphosphatidylinositol (GPI) which associated with host cell attachment (Tomavo et al., 1989; Manger et al., 1998). A previous report also showed that targeted disruption of GPI anchored TgSAG3 decreased parasite invasive ability (Dzierszinski et al., 2000). TgSRS29B (formerly TgSAG1), is an important surface adhesin that can bind host cell (Wasmuth et al., 2012). Consistent with these reports, deletion of TgSRS2 resulted in attenuated infectivity in PLK strain in this study, which could be associated with its glycosylphosphatidylinositol structure.

Remarkably, loss of TgSRS2 resulted in a dramatic attenuation of PLK strain virulence in mice. Similarly, disruption of TgSAG3 or TgSRS29B also showed a markedly reduced capacity to cause mortality in mice. Thus the decreased virulence may be due to a reduction of the invasion ability, or an altered host immune response. Interestingly, the transoverexpression of type II TgSRS2 in RH strain, dramatically

decreased its virulence in mice (Wasmuth et al., 2012). An opposite role of TgSRS2 was observed in RH and PLK strains. These results suggest that TgSRS2 may act in different pathway in the two strains and the phenotypes of the two strains could be caused by deletion and overexpression of TgSRS2 together with other proteins.

Pszenny found that high levels TgSRS2 protein of RH strain dramatically reduced the levels of gamma interferon (IFN-γ) at the peak of *in vivo* infection (Wasmuth et al., 2012). Acute infection usually causes the dysregulation of IFN-γ and interleukin-12 leading to severe immunopathology and death (Gavrilescu and Denkers, 2001; Mordue at al., 2001). Thus, TgSRS2 antigen may play a role in fine-tuning or negatively regulating the proinflammatory effector to alter cytokine induction and/or the signaling pathway resulting in dysimmunity with other molecules of RH parasite or host cell (Rachinel et al., 2004; Saeij et al., 2006; Saeij et al., 2007; Peixoto et al., 2010). However, there is no report on the role of TgSRS2 in immune response of the host to PLK strain infection. Therefore, further studies should be performed to explore the mechanism through which TgSRS2 regulates the virulence of different *T. gondii* strains in mice.

1-5. Summary

T. gondii is an opportunistic pathogenic protozoon, it can cause abortion in pregnant women and cause fetal congenital diseases, it's also fatal in immunodeficient individuals. Cellular invasion by *T. gondii* is an active complex multistep event. The process is initiated by contact between the parasite and the cell surface of the host. The surface antigens of *T. gondii* are important for attachment to host cells in the process of *T. gondii* infection. TgSRS2 is differentially expressed

among different *Toxoplasma* genotypes, although its function remains unknown. In this study, I used CRISPR-CAS9 system to generate knockout SRS2 gene in *T. gondii* RH and PLK strains. Phenotype analysis revealed that TgSRS2 knockout significantly inhibited the invasion, but did not affect the replication of *T. gondii* PLK genotype. Mouse experiments demonstrated that PLK TgSRS2 knockout parasites significantly reduced the pathogenicity of *T. gondii* in mice. However, deficiency of TgSRS2 in RH genotype did not have any effect on invasion, replication and virulence *in vivo*. Overall, TgSRS2 of PLK strain may play an important role in host cell invasion and pathogenicity in mice. This study helps to better understand the function of TgSRS2.

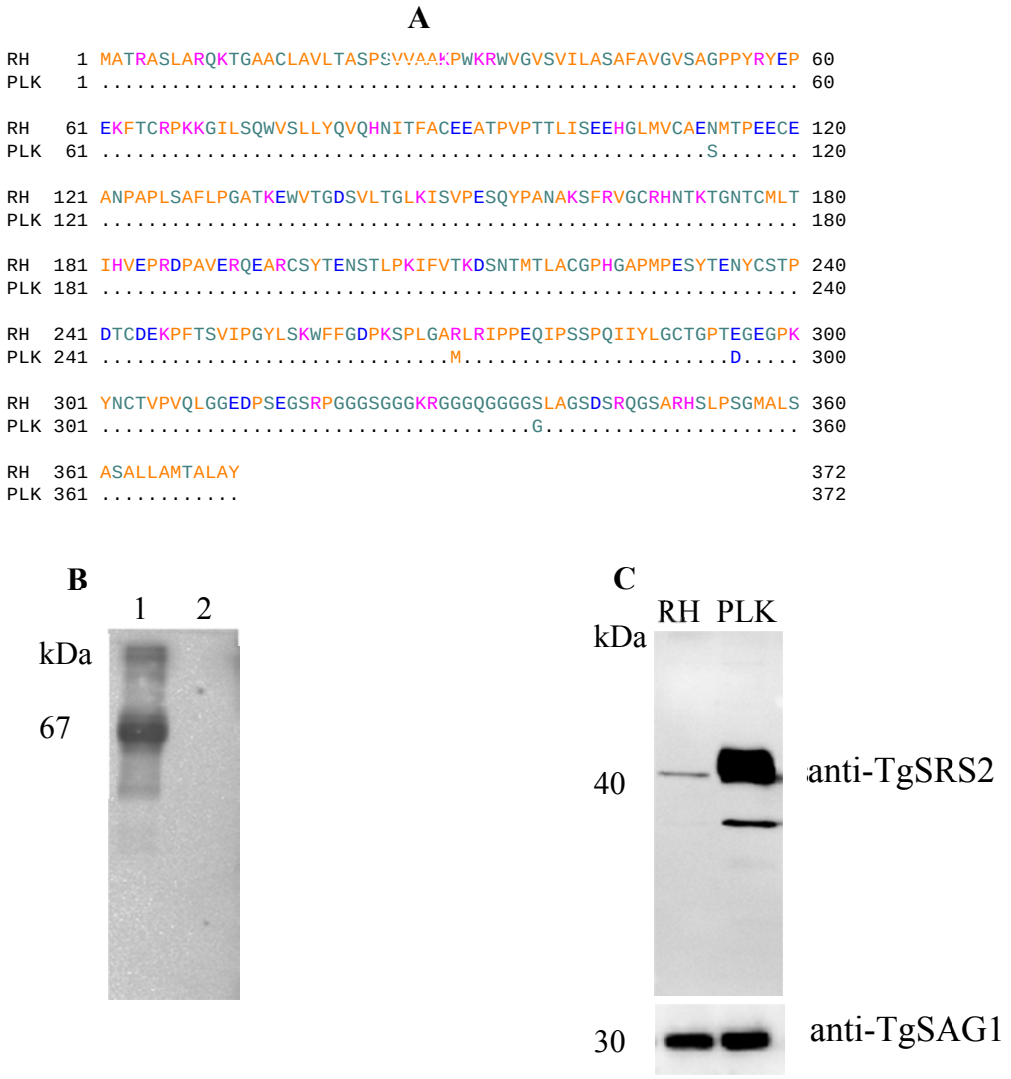

Fig. 1. Western blot analysis. (A) The predicted amino acid sequences for the primary translation product of the TgSRS2 gene in two *T. gondii* strains. (B) Western blot analysis of rTgSRS2 protein. Lane **1**, Purified rTgSRS2 incubated with serum from mice experimentally infected with *T. gondii*. Line 2, Purified rTgSRS2 incubated with a serum from healthy mouse. (C) Tachyzoite lysates of RH and PLK strains incubated with anti-TgSRR2 serum from mice.

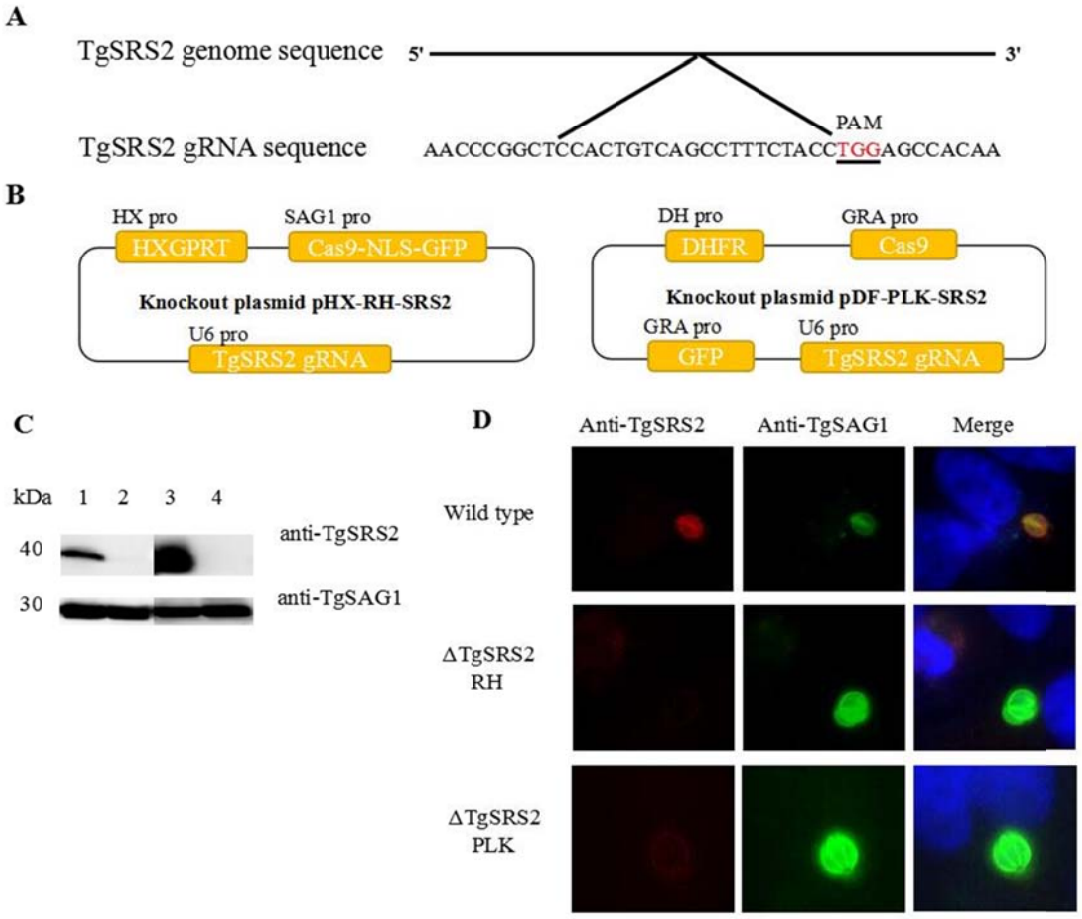

Fig. 2. TgSRS2 knockout in RH and PLK strains. (A) TgSRS2 gRNA sequence and locus in TgSRS2 genomic sequence. PAM, protospacer-adjacent motif. (B) Schematic illustration of knockout plasmids. Both plasmids contain four important genes: CAS9, TgSRS2 gRNA, GFP and hypoxanthine xanthine guanosine phosphoribosyl transferase (HXGPRT)/dihydrofolate reductase (DHFR). The genes' promoters (pro) are labeled. NLS, nuclear localization signal. (C) Western blot analysis. TgSRS2 and TgSAG1 were detected using mice anti-TgSRS2 and mice anti-TgSAG1 sera, respectively. Lane 1, wild type RH strain; Line 2, ΔTgSRS2 of RH strain; Line 3, wild type PLK strain; Line 4, ΔTgSRS2 of PLK strain. (D) IFAT analysis. ΔTgSRS2 was detected by mice anti-TgSRS2 serum (red).

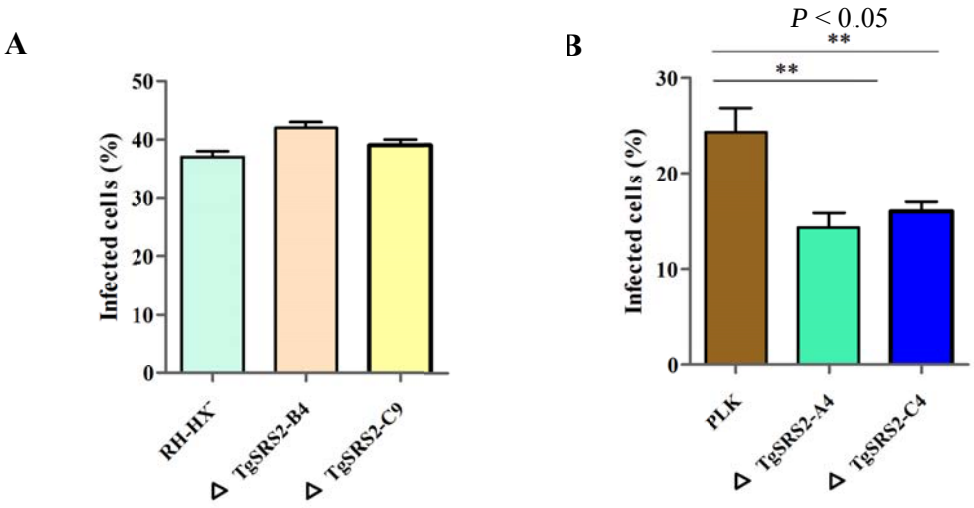

Fig. 3. Deletion of TgSRS2 affects host cell invasion *in vitro*. (A, B) The percentage of cells invaded and re-invaded by equal number tachyzoites of each strain (2×10^5/well) was calculated by IFAT. After the parasites were allowed to invade 2 h, extracellular parasites were washed away with PBS. After further 22 h of growth, IFAT was performed. Results represent means ± SEM of triplicates. **, $P < 0.05$, determined by one-way ANOVA Tukey's multiple comparison test.

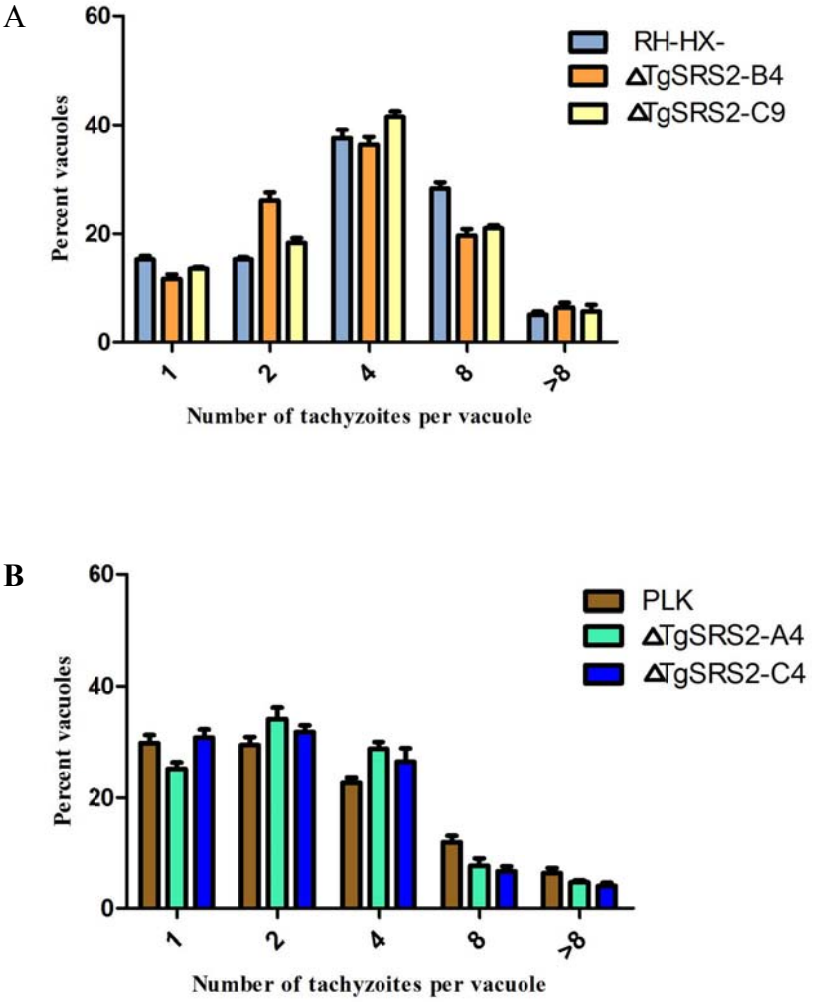

Fig. 4. Deletion of TgSRS2 does not affect parasite growth *in vitro*. (A, B) Replication of each strain was evaluated by IFAT. At least 100 vacuoles were counted in each well, in each condition. Error bars represent mean percent values ± SEM of triplicates.

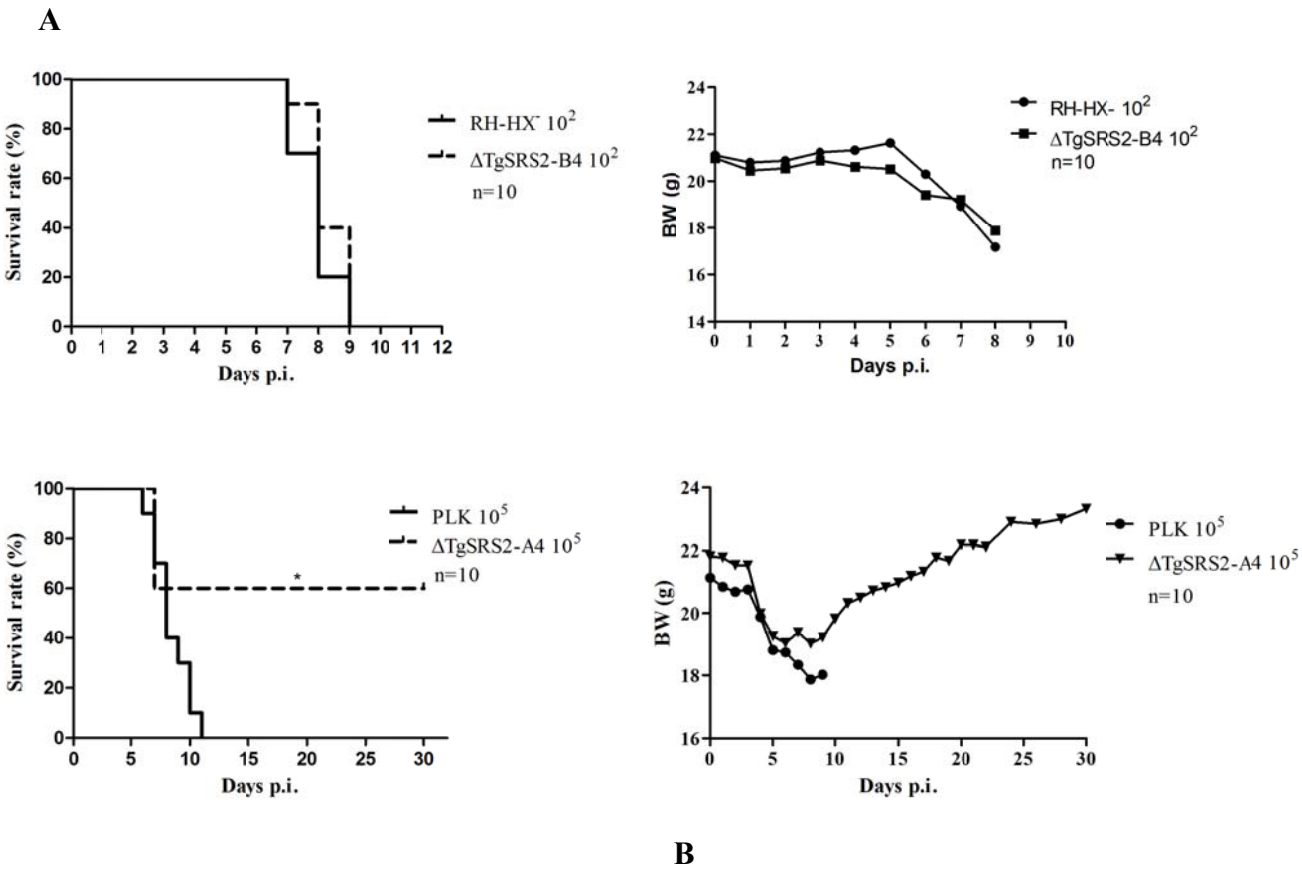

Fig. 5. Loss of TgSRS2 affects parasite virulence in mice. Survival rate (A) and body weight (B) of mice infected with RH and ΔTgSRS2-B4 strains. Survival rate (C) and body weight (D) of mice infected with PLK and ΔTgSRS2-A4 strains. *, P = 0.0197, determined by the long-rank test.

C **D**

Chapter 2

Development and evaluation of an enzyme-linked immunosorbent assay based on recombinant TgSRS2 for serodiagnosis of *Toxoplasma gondii* infection in cats

2-1. Introduction

Toxoplasmosis in animals has a significant reproductive and economic impact, especially in sheep and goats (Buxton, 1998). Development of accurate diagnostic methods is crucial for proper management and control of *T. gondii* infection in humans and animals. A number of methods have been developed. They include histologic demonstration, isolation of the organism, amplification of specific nucleic acid sequences and serological tests (Montoya, 2002). The latex agglutination test (LAT) is considered the "gold standard" for detecting *T. gondii* infection, but has low specificity and high cost (Dannemann, 1990). Enzyme-linked immunosorbent assays (ELISAs) seem to be the most suitable for routine mass diagnosis such as in seroepidemiological investigations (Montoya, 2002). The specificity and sensitivity of these serologic methods depend mostly on the diagnostic antigen used.

Several recombinant proteins hold potential to be used for *Toxoplasma* diagnosis in cats and humans, and among them major surface antigens (TgSAGs) and dense granule antigens (TgGRAs) have been shown to be good candidates (Kotresha and Noordin, 2010; Khanaliha et al., 2014; Cai et al., 2015; Abdelbaset et al., 2017). SAG1-related superfamily (TgSRS) proteins are expressed in a stage-specific manner, are essential for entry into the host cell, and regulate host immunity to promote chronic infection (Jung et al., 2004). SRS29B (TgSAG1) and SRS34A (TgSAG2A) have been used in indirect ELISA for serodiagnosis of feline toxoplasmosis (Kimbita et al., 2001; Huang et al., 2002). TgSRS2 has been identified by Manger in 1998, however its antigenicity is unknown. In the present study, I describe the performance of TgSRS2 for application in serological surveys.

2-2. Materials and methods

Parasites and host cells

The *T. gondii* RH (Type I strain) and PLK (Type II strain) tachyzoites were grown *in vitro* on human foreskin fibroblast (HFF) cell monolayers in minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin-streptomycin. The cultures were incubated at 37°C in a 5% CO_2 atmosphere.

Mice

ICR (6-7 weeks of age) were purchased from Clea Japan (Tokyo, Japan) and housed in the mouse room of the National Research Center for Protozoan Diseases under the Guiding Principles for the Care and Use of Research Animals promulgated by the Obihiro University of Agriculture and Veterinary Medicine.

Purification of TgSRS2 and GST

pGEX-TgSRS2 plasmid has been constructed in previous study (chapter 1). In the current study, pGEX-TgSRS2 and pGEX-4T-1 (plasmid without insert) were expressed as a glutathione-S-transferase (GST) fusion protein in *E. coli* strain BL21 strain. The expression was achieved at 16°C for 24 h after induction with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The *E. coli* culture was centrifuged at 10,000 × g for 10 min, and the pellet was suspended in STE buffer (50 mM Tris-HCl [pH 9.5], 1 mM EDTA [pH 8.0] and 150 mM NaCl). Triton X-100 was added to the samples to a final concentration of 1% (w/v) Triton X-100, and the samples were sonicated on ice for 10 min. The supernatant that contained the soluble fraction was purified with Glutathione Sepharose 4B beads, according to the manufacturer's protocols (GE Healthcare, UK). The bound GST-fused recombinant proteins were eluted with the elution buffer (16 mM reduced glutathione , 100 mM Tris-HCl [pH 8], 5 mM EDTA, 100 mM NaCl). The amount of recombinant TgSRS2 and GST were measured using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the concentrations were measured by bicinchoninic acid (BCA) protein assay kit (Thermo Fisher, USA) according to the manufacturer's protocol (Pierce Biotechnology, USA).

ELISA

MaxiSorp plates (Nunc, Denmark) were coated with each recombinant antigen (TgSRS2, GST) at a final concentration of 1 μg/ml in a coating buffer (50 mM carbonate, pH 9.6) and were incubated overnight at 4°C. Plates were blocked with 3% skim milk in phosphate-buffered saline for 1 h at 37°C. Tested sera were diluted at 1:100 and were added to the wells and incubated for 1 h at 37°C. Plates were washed 6 times and incubated at 37°C for 1 h with horseradish peroxidase (HRP)-conjugated

goat IgG (Thermo Fisher, USA) diluted at 1:5,000. After washing, 100 µl of substrate solution [0.1 Mcitrate buffer, pH 4, 0.003% H_2O_2 and 0.3 mg/ml 2, 20-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); Sigma-Aldrich, USA] was added to each well. The absorbance was measured at 415 nm using an ELISA reader (Corona micro plate reader MTP-120; Corona, Japan) after 1 h of incubation at RT. Each sample was examined at least twice. Sera collected from mice before parasite infection served as negative controls.

Sera

To prepare *T. gondii*- and *N. caninum*-infected sera, 7 week-old ICR female mice were intraperitoneally inoculated with 10^3 tachyzoites of *T. gondii* PLK or 10^4 tachyzoites *N. caninum*. Sera were collected every week until the 12^{th} week post infection. Tested sera consisted of 76 clinical serum samples and 32 SPF serum samples were obtained from Clinical Laboratory Department of Malpie - Lifetech Inc in Osaka. The serum samples were stored at -30°C and later used for testing TgSRS2 based ELISA. The cutoff value of each recombinant antigen was calculated as the average OD of 32 SPF cat serum samples plus 3 standard deviations.

LAT

Antibodies to *T. gondii* were detected using a latex agglutination test (LAT) kit (Toxocheck®-MT, Eiken Chemical, Japan) according to the manufacture's instructions. In brief, the LAT was carried out in a 96-well U bottomed plate with two-fold dilutions from 1:16 to 1:2,048. The plate was slightly shaken for about 2 min and then incubated at room temperature for at least 18 hours without shaking. Test samples at a dilution rate of at least 1:64 that showed a layer of agglutinated latex beads after incubation were considered positive.

Statistical analysis

The results of ELISA and LAT were used to evaluate sensitivity, specificity, percentage of agreement, and the kappa values of the new diagnostic test (http://vassarstats.net/).

2-3. Results

Expression of rTgSRS2 and GST in *E.coli*

The molecular masses of rTgSRS2 and GST proteins were estimated as 67 kDa and 26 kDa, respectively, as expected (Fig. 6A).

Evaluation of the rTgSRS2 based ELISA using experimentally infected mice

To evaluate the potential use of rTgSRS2 for diagnosis of *T. gondii* infection, ELISA based on recombinant proteins was performed. The ELISA based on rTgSRS2 succeeded in clearly differentiating *T. gondii*-infected mouse sera and non-infected mouse sera or *Neospora caninum*-infected sera (Fig. 7A). Furthermore, to determine the sensitivity of the developed ELISA, *T. gondii*-infected mouse sera were serially sampled post-infection (Fig. 7B). rTgSRS2 based ELISA could detect specific antibody starting from the 3rd week after infection.

Evaluation on field cat samples

The developed ELISA assays were evaluated on cat samples. Among the 76 cat samples, rTgSRS2 based ELISA detected *T. gondii*-specific antibodies in 49 (64.5%) samples. Next, the performance of the ELISA was compared to LAT as a reference (Table 1). The sensitivity and specificity of rTgSRS2 ELISA were 95% and 71%,

respectively. ELISA based on rTgSRS2 showed high concordance (84%) and substantial agreement (kappa value > 0.61) with the results of LAT (Table 2).

2-4. Discussion

Toxoplasmosis is one of the most common zoonotic diseases and remains a worldwide public health problem (Hill et al., 2013). As the most common definitive host, cats play an extremely important role in the transmission of *T. gondii* (Webster, 2007). Therefore, for control of toxoplasmosis it is necessary to develop an accurate method to detect *T. gondii* infection in cats.

In the present study, the performances of ELISA employing rTgSRS2 was evaluated in sera of *T. gondii* experimentally infected mice and clinical cats. The lack of reactivity with normal or *N. caninum*-infected mouse sera indicates the specificity of the rTgSRS2 based ELISA. rTgSRS2 ELISA succeeded in the detection of antibody to *T. gondii* in the acute and chronic stages. Detection of antibodies to *T. gondii* in the chronic stage may contribute to the diagnosis of disseminated toxoplasmosis that could be reactivated by immune-suppression due to viral infection or cancer (Kimbita et al., 2001).

In the field cat samples, rTgSRS2 demonstrated a good diagnostic performance. Hence, the higher positive rate of rTgSRS2 may indicate that some of the samples are from chronically infected cats, and also suggests that rTgSRS2 has good antigenicity. The antigenic properties of rTgSRS2 can be explained by the fact that this protein is an important regulator of virulence capable of the development of protective immunity (Wasmuth et al., 2012). rTgSRS2 ELISA has higher sensitivity but lower

specificity than previously reported rTgSAG2, rTgGRA7 and rTgGRA14 based ELISA (Terkawi et al., 2013).

Although LAT shows the advantage that it can be used in serum samples from various animals, it does not provide information on the stage of infection as it does not detect specific IgM to *T. gondii* in feline sera (Lappin et al., 1991). Antigens of bacterial recombinant proteins have many advantages in diagnosis, with low cost of production and better standardization of the tests (Pietkiewicz et al., 2004). In the current study, recombinant protein TgSRS2 showed good antigenicity and reactivity with mouse and cat serum samples when tested as an ELISA antigen. My results suggest that ELISA with rTgSRS2 could be a useful diagnostic tool for detection of *T. gondii* infection in animals.

2-5. Summary

Toxoplasmosis is an important zoonosis widely prevalent in humans and animals. To develop a specific and sensitive serodiagnostic assay, the SAG1-related sequence 2 of *T. gondii* (TgSRS2) was expressed in *Escherichia coli* and its diagnostic potential was evaluated in an enzyme-linked immunosorbent assay (ELISA). The specificity and sensitivity of recombinant TgSRS2 (rTgSRS2) was confirmed in the ELISA using a series of mouse serum samples. The results showed that the ELISA based on rTgSRS2 could detect specific antibodies in both acute and chronic infections. A total of 76 clinical cat samples were also examined by the ELISA based on rTgSRS2, and the results were compared with those of the latex agglutination test (LAT). rTgSRS2 had a higher rate of positivity, with concordance (84%) and kappa value (0.68) in comparison with the results using LAT. The ELISA-rTgSRS2 showed high sensitivity

(95%) but low specificity (71%). This is the first report evaluating TgSRS2 as a serodiagnostic antigen. These results suggest that the ELISA based on rTgSRS2 could be a useful tool for routine testing in the clinic and mass screening of samples in the field.

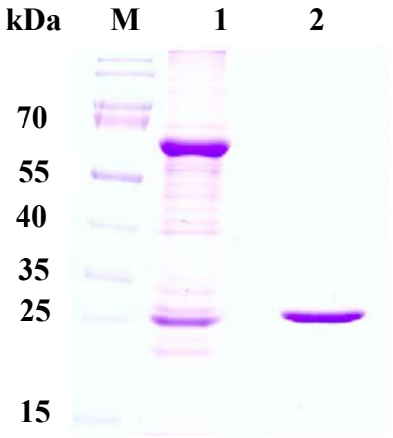

Fig. 6. SDS-PAGE of expression of recombinant proteins *in E. coli*. M, molecular marker; Lanes 1, rTgSRS2; Lanes 2, GST.

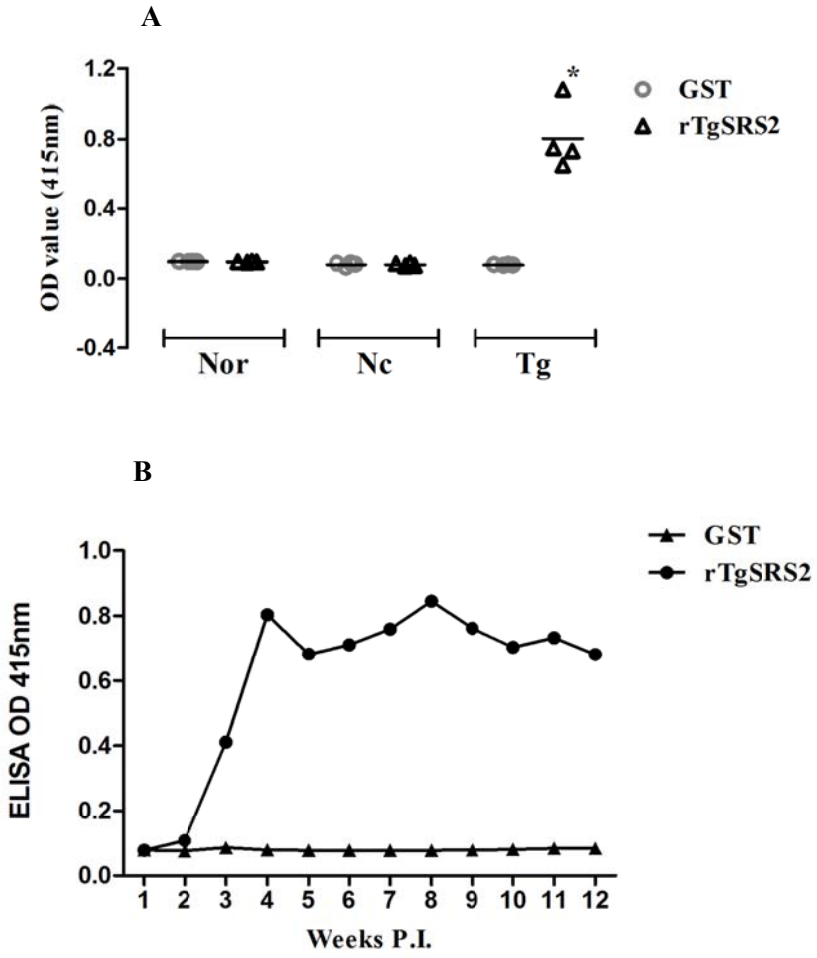

Fig. 7. Detection of antibodies to *T. gondii* by ELISA using recombinant antigens in the sera of experimentally infected mice. (A) The reactivity of rTgSRS2 and GST with sera from normal mice, experimentally *T. gondii*-infected mice and experimentally *N. caninum*-infected mice at the 4th week after infection were evaluated by ELISA (n = 4). (B) The reactivity of recombinant antigens with experimentally *T. gondii*-infected mouse sera collected weekly (1th-12th).

Table 1. Comparison of the results for *T. gondii* antibodies in cat samples examined by ELISA and LAT.

	ELISA＋	ELISA－	Total
LAT＋	39 (51.3%)	2 (2.6%)	41 (53.9%)
LAT－	10 (13.2%)	25 (32.9%)	35 (46.1%)
Total	49 (64.5%)	27 (35.5%)	76 (100%)

Table 2. Specificity and sensitivity of ELISA with recombinant protein in detection of specific *Toxoplasma* antibodies in cats compared with the results in LAT.

Detection method	Sensitivity (%)	Specificity (%)	Concordance (%)	Kappa value
rTgSRS2-ELISA	95	71	84	0.68

Chapter 3

Seroprevalence of *Toxoplasma gondii* infection in sheep from northern China

3-1. Introduction

Toxoplasma gondii can infect animals and humans via three pathways: ingestion of oocysts from the environment, ingestion of tissue cysts in undercooked meat, and congenital transmission from an infected mother to the fetus during pregnancy (Montaya and Liesenfeld, 2004; Dubey, 2010). Ingestion of raw or undercooked meat of animals harboring tissue cysts is considered the most important source of human infection (Hill and Dubey, 2015). Thus, humans can get infected with *T. gondii* by ingestion of undercooked lamb and mutton. As many people have the habit of eating undercooked 'barbecue' and 'instantly boiled mutton' in China, the risk of contracting *T. gondii* infection constantly exists.

In recent years, a growing number of surveys of *T. gondii* infection in sheep have been carried out worldwide (Gebremedhin et al., 2014; Hammond-Aryee et al., 2015; Tegegne et al., 2016; Özmutlu and Karatepe., 2017), and several surveys have shown that *T. gondii* infection in sheep is common in some provinces of China (Wu et al., 2011; Wang et al., 2011; Xu et al., 2015; Liu et al., 2015; Yin et al., 2015; Zou et al.,

2015). However, there is a gap in knowledge about *T. gondii* infection in sheep in northern China. Considering the public health importance and economic significance of products from sheep, it is essential to determine the prevalence of *T. gondii* infection, and to develop a specific and sensitive serodiagnostic method in sheep.

As a reference, latex agglutination test (LAT) is widely used in detection of *T. gondii* antibody in different animal species, but has a high cost (Dannemann, 1990; Huang et al., 2002; Shahiduzzaman et al., 2011; Kyan et al., 2012; Matsuo et al., 2014). *T. gondii* SAG1-related sequence 2 (TgSRS2) is a surface antigen expressed in the tachyzoite stage of the parasite (Manger, 1998). In my previous study, this protein showed good antigenicity and high sensitivity when used as an antigen to detect the *T. gondii* antibody in cats (chapter 2). In the current study, a survey was performed to evaluate the seroprevalence of *T. gondii* infection in sheep from northern China and to assess the risk factors for infection using LAT and an enzyme-linked immunosorbent assay (ELISA) based on rTgSRS2 protein.

3-2. Materials and methods

Study areas and animals

A total of 288 blood samples were collected from asymptomatic sheep in June, 2017 in four northern provinces of China (Fig. 8), namely Chifeng, Inner Mongolia (n = 76), Jiamusi, Heilongjiang (n = 80), Siping, Jilin (n = 65) and Tangshan, Hebei (n = 67). Age, gender and rearing system details were recorded. Animals were reared in extensive and intensive systems for meat and were generally kept in herds of 50-100 and more than 100 animals, respectively. Approximately 5 ml of blood was obtained from the jugular vein of each sheep, then serum samples were separated after

centrifuging at 3,000 × g for 5 min and stored at -20°C until tested for antibodies to *T. gondii*.

Serological examination

Antibodies to *T. gondii* were detected using a LAT kit (Toxocheck®-MT, Eiken Chemical, Japan) according to the manufacturer's instructions and ELISA based on rTgSRS2 where GST protein was used as a control. In brief, the LAT was carried out in a 96-well U bottomed plate with two-fold dilutions from 1:16 to 1:2,048. The plate was slightly shaken for about 2 minutes and incubated at room temperature for least 18 hours without shaking. Test samples at a dilution rate of at least 1:64 that showed a layer of agglutinated latex beads after incubation were considered positive. The ELISA was carried out using methods from chapter 2. Twenty sheep serum samples which were seronegative tested by LAT and Western blot were used to calculate the cutoff value of ELISA.

Statistical analysis

T. gondii seroprevalence per age groups, rearing systems, gender and different locations was calculated and compared using the 95% Confidence Interval (CI) of Odds Ratio (OR) (http://www.vassarstats.net/). The differences were considered statistically significant if $P < 0.05$.

3-3. Results

The *T. gondii* antibody titers by LAT in 288 sheep are presented in Table 3. Antibody titers in positive samples ranged from 1:64 to 1:1,024. These samples were

also screened by ELISA method using rTgSRS2 with GST tag (Table 2). Overall, 87 (30.2%), 101 (35.1%) and 73 (25.5%) samples were positive for anti-*T. gondii* antibodies according to LAT, ELISA, and both tests, respectively. The seroprevalence in Hebei were the highest at 46.3%, 53.7% and 38.8%; followed by Heilongjiang at 33.8%, 38.8% and 28.8%; Jilin at 24.6%, 29.2% and 20%; Inner Mongolia at 17.1%, 19.7% and 14.5% according to LAT, ELISA, and both tests, respectively (Table 4). In addition, significant difference was observed between Inner Mongolia and Hebei.

The seroprevalence of *T. gondii* was 12.3% in the age group of 0-1 year, 25.5% in 1-2 years and 34.1% in > 2 years. Sheep of less than 1 year were less likely to be seropositive than those of 1-2 and those of > 2 years, and the difference was statistically significant (Table 4).

The seroprevalence in female sheep (26.9%) was slightly higher than in male sheep (22.1%), although the difference was not statistically significant (Table 4). With regards to the rearing system, the seroprevalence in sheep raised intensively (20.4%) was significantly lower than in those raised extensively (31.3%) (Table 4).

The recombinant protein TgSRS2 was used as antigen for ELISA to evaluate its diagnostic performance with field sheep sera. The result of ELISA with rTgSRS2 showed higher positive rate than LAT for the detection of *T. gondii* antibodies (Table 4). The kappa value of rTgSRS2 was 0.67, which demonstrates substantial agreement between the rTgSRS2 ELISA and LAT (Table 6).

3-4. Discussion

The present study investigated the presence of antibodies against *T. gondii* in sheep from Hebei, Heilongjiang, Jilin and Inner Mongolia, Northern China. Both the

LAT and TgSRS2-based ELISA results showed that anti-*T. gondii* antibodies are prevalent in the 4 provinces with significant difference between Inner Mongolia and Hebei. There are two likely reasons for this notable difference. Firstly, the winter season is nearly 5 months and is longer in Inner Mongolia than in Hebei, and the oocysts of *T. gondii* excreted from cat faeces can hardly become infective under low temperatures. Secondly, the seroprevalence of *T. gondii* in its definitive hosts cats is significantly higher (57.3%) in Hebei than in Inner Mongolia (10.3%) (Ding et al., 2017), which may be a major risk factor for the high infection in Hebei, as cats play a significant role in *T. gondii* transmission (Dubey and Schares, 2011).

The overall seroprevalence of *T. gondii* (25.3%) obtained in the present study was far lower than in Bangladesh (69.9%) (Rahman et al., 2014) and India (50%) (Singh et al., 2015), but higher than in some other provinces of China (Table 5). These differences could be associated with the detection methods, age distribution of the samples, sample sizes, rearing systems, ecological conditions and climates in each investigation. However, all these reports provide strong evidence that *T. gondii* infection is common in sheep in China, and could cause considerable economic losses and pose health risk in both animals and humans.

The seropositivity for *T. gondii* was significantly related to the age of the sheep. This result agrees with previous reports (Ramzan et al., 2009; Xu et al., 2015), and a similar association has been reported in other species such as cattle (Qiu et al., 2012). This suggests the possibility of horizontal transmission in the investigated regions and provides evidence for the increased infection risk for older animals, as they have a longer period to potentially be exposed and infected by *T. gondii*. Animal gender did not affect the seropositivity to *T. gondii* whereas rearing system was a significant risk factor. This result is in accordance with a previous investigation in Shenyang province

of China (Xu et al., 2015), indicates that the gender has no significant effect on the prevalence of *T. gondii* in sheep. The effects of rearing system were also reported in Heilongjiang and Liaoning provinces of China (Wang et al., 2011; Xu et al., 2015). The intensive system of raising sheep implements strict hygiene practices, including restriction of animal movement. Thus, they have fewer chances to contact or ingest the oocysts of *T. gondii* excreted by infected cats.

Previously, TgSRS2 showed potential as diagnostic antigen for the detection of antibodies of toxoplasmosis in domestic cats (chapter 2). The ELISA based on rTgSRS2 can clearly differentiate the samples between *Neospora caninum*-infected sera, non-infected mouse sera and *T. gondii*-infected mouse sera. The sensitivity of the TgSRS2 ELISA was determined by serial *T. gondii*-infected sera, and the specific antibody could be detected as early as the third week after infection. Furthermore, high concordance (85%) and substantial agreement of the ELISA based on TgSRS2 also demonstrated that TgSRS2 is a promising antigen in detecting *T. gondii* antibodies. This is the first report to use TgSRS2-based ELISA to detect antibodies of toxoplasmosis in sheep. The results of TgSRS2-based ELISA highly matched with the results of LAT, with high kappa value and concordance, suggesting it is a potential diagnostic tool for routine screening against toxoplasmosis.

In conclusion, the present survey revealed that antibodies to *T. gondii* are widespread in sheep in northern China, which has great significance for both livestock economy and public health. In addition, it can be inferred that extensive rearing system is a potential risk factor for *T. gondii* infection in China. These data could provide a foundation for prevention and control of *T. gondii* in sheep in the examined provinces.

3-5. Summary

Toxoplasma gondii is an important zoonotic parasite causing significant health problems to humans and animals. In the recent years, a number of investigations about the seroprevalence of *T. gondii* in China have been reported, but little is known on the prevalence of toxoplasmosis in sheep in northern China. In the present study, a total of 288 sheep serum samples were collected from Inner Mongolia, Heilongjiang, Jilin and Hebei provinces of northern China for *T. gondii* antibody survey using LAT and TgSRS2-based ELISA. Overall, 87 samples (30.2%) of 288 serum samples were positive for antibodies to *T. gondii* from the northern China. Seroprevalence of *T. gondii* infection in sheep was 53.7% in Hebei, 38.8% in Heilongjiang, 29.2% in Jilin and 19.7% in Inner Mongolia according to ELISA. Age and rearing system significantly affected seropositivity. The present survey indicates that antibodies to *T. gondii* are widely prevalent in sheep in northern China, and these results will be useful as surveillance data for toxoplasmosis in animals and humans in the study area.

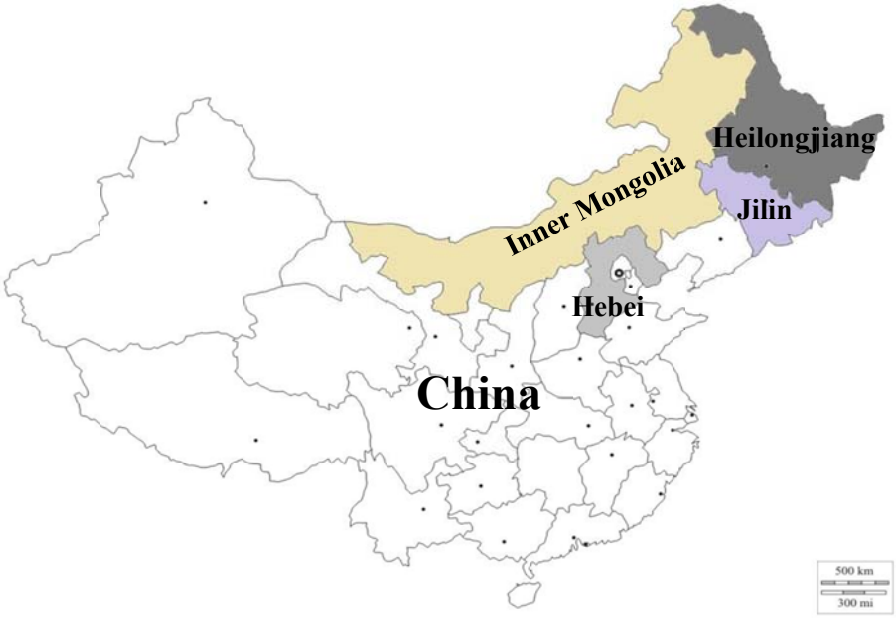

Fig. 8. Geographic distribution of the sampling sites used.

Table 3. *T. gondii* antibody titers, by latex agglutination test (cut-off ≥ 64) in 288 sheep sera.

Titer	No. of sample (%)	Results by LAT
≤1:32	201 (69.8)	−
1:64	32 (11.1)	+
1:128	17 (5.9)	+
1:256	14 (4.9)	+
1:512	19 (6.6)	+
1:1024	5 (1.7)	+

+ : positive, − : negative

Table 4. Comparison of the results for *T. gondii* antibodies in sheep samples examined by ELISA and LAT.

Characteristics	LAT		ELISA	LAT/ELISA	Odd ratio	
	No. of tested	No. of positive(%)	No. of positive(%)	No. of positive(%)	(OR) (95% CI)	P-value
Location						
Inner Mongolia	76	13 (17.1)	15 (19.7)	11 (14.5)	Reference	
Heilongjiang	80	27 (33.8)	31 (38.8)	23 (28.8)	0.40 (0.19-0.86)	0.014
Jilin	65	16 (24.6)	19 (29.2)	13 (20.0)	0.63 (0.28-1.44)	0.19
Hebei	67	31 (46.3)	36 (53.7)	26 (38.8)	0.24 (0.11-0.52)	<0.001
Age (year)						
0-1	57	8 (14.0)	9 (15.8)	7 (12.3)	Reference	
1-2	149	46 (30.9)	54 (36.2)	38 (25.5)	0.37 (0.16-0.83)	0.009
>2	82	33 (40.2)	38 (46.3)	28 (34.1)	0.24 (0.10-0.58)	<0.001
Gender						
Male	95	25 (26.3)	29 (30.1)	21 (22.1)	Reference	
Female	193	62 (32.1)	72 (37.3)	52 (26.9)	0.75 (0.44-1.30)	0.19
Rearing system						
Extensive	131	49 (37.4)	57 (43.5)	41 (31.3)	Reference	
Intensive	157	38 (24.2)	44 (28.0)	32 (20.4)	1.87 (1.13-3.11)	0.011
Total	288	87 (30.2)	101 (35.1)	73 (25.3)		

Table 5. Reported prevalence rates of *T. gondii* infection in sheep in China.

Provinces/cities	Year of sampling	No. tested	Positive (%)	Serologic test[a]	References
Heilongjiang	2008-2010	792	3.0	IHA	Wang et al., 2011
Liaoning	2011	566	4.4	IHA	Yang et al., 2013
Liaoning (Jinzhou)	2012	402	17.9	MAT	Xu et al., 2015
Qinghai (Tibet)	2011	455	5.7	IHA	Wu et al., 2011
Qinghai	2012-2013	600	21.3	ELISA	Liu et al., 2015
Gansu	2013-2014	1732	20.3	MAT	Yin et al., 2015
Yunnan	2012-2013	154	9.7	IHA	Zou et al., 2015
Henan	2015-2016	779	12.7	MAT	Zhang et al., 2016

[a] MAT: modified agglutination test, ELISA: enzyme-linked immunosorbent assay, IHA: indirect hemagglutination assay

Table 6. Specificity and sensitivity of ELISA with recombinant protein compared to LAT for detection of *Toxoplasma* antibodies in sheep.

Detection method	Sensitivity (%)	Specificity (%)	Concordance (%)	Kappa value
rTgSRS2-ELISA	84	86	85	0.67

General discussion

Toxoplasma gondii is an obligate intracellular protozoan parasite belonging to the phylum Apicomplexa. It can infect most warm-blooded animals and has a worldwide distribution (Tenter et al., 2000). Scholars estimate that about 200,000 new cases of toxoplasmosis occur every year. There are no clear symptoms of toxoplasmosis in healthy adults. However, toxoplasmosis is life threatening in immunocompromised individuals (Jones et al., 2003). The increasing emergence of toxoplasmosis has resulted in demands for effective diagnostic methods and urgent preventive strategies to detect and control the disease. In the current study, in order to better understand the function of TgSRS2, I generated ΔTgSRS2 parasites using CRISPR/CAS9 technology both in RH and PLK strain, and analyzed the change in their phenotypes. TgSRS2 may not be crucial for the development of the parasite because the mutant parasites didn't show any defect in replication *in vitro.* Loss of TgSRS2 in PLK strain dramatically reduced the efficiency of the invasion and virulence in mice, but this was not observed in RH mutant strain. In a previous study, TgSRS2 showed a higher expression level in the avirulent type II than the virulent type I (Manger et al., 1998). In transgenic parasites, TgSRS2 overexpression in type I or type II RH strain can significantly reduce virulence in mice (Wasmuth et al., 2012). This data indicate that the expression level of TgSRS2 is an important key to regulate its function in *T. gondii*. Like other surface proteins, TgSRS2 has an N-terminal signal peptide and is anchored with the GPI which associated with host cell attachment (Tomavo et al., 1989; Manger et al., 1998). Previous reports showed that TgSRS29B and TgSAG3 are important surface adhesin that can bind to host cell (Dzierszinski et

al., 2000; Wasmuth et al., 2012). Thus the attenuated infectivity of ΔTgSRS2 in PLK strain could be associated with its GPI structure.

In animal experiment, all mice infected with 100 tachyzoites of ΔTgSRS2 RH or parental strains succumbed within 7-9 days post-infetcion. Interestingly, mice infected with 10^4 tachyzoites of ΔTgSRS2 PLK showed 100% survival rate while only 60% survival rate was observed when infected with the same dose of the PLK strain. Moreover, mice infected with 10^5 tachyzoites ΔTgSRS2 PLK also showed significantly higher survival rate than those infected by the PLK strain. The results indicate that TgSRS2 may be an important virulence factor. The reduced invasive ability of ΔTgSRS2 PLK might have contributed to the higher survival rate. The similarity in pathogenicity between ΔTgSRS2 RH and parental RH strains could be due to the little expression of TgSRS2 in RH (Manger et al., 1998). A previous study proved that TgSRS2 was a regulator of RH strain virulence (Wasmuth J D et al., 2012). This suggests that TgSRS2 may act in different pathways in the two strains. High levels of TgSRS2 protein in RH strain dramatically reduced the levels of IFN-γ at the peak of the *in vivo* infection (Wasmuth J D et al., 2012). Usually acute infection can cause the dysregulation of IFN-γ leading to severe immunopathology and death (Gavrilescu LC, Denkers EY., 2001; Mordue DG at al., 2001). Therefore, TgSRS2 protein may play a role in fine-tuning or negatively regulating the proinflammatory effector to alter cytokine induction and/or the signaling pathway resulting in dysimmunity with other molecules of RH parasite or host cell (Rachinel N, et al., 2004; Peixoto L, et al., 2010; Saeij JP, et al., 2006; Saeij JP, et al., 2007). However, no study has reported on the immunoreaction of the host against PLK strain in relation to TgSRS2.

As a major surface antigen, TgSRS2 was used as a diagnostic antigen for ELISA to develop a specific and sensitive serological diagnosis for toxoplasmosis in domestic cats. The cat is the definitive host of *T. gondii* and plays an important role in transmission of toxoplasmosis (Dubey and Schares, 2011). This was the first use of rTgSRS2 antigen in detection of antibodies against *T. gondii*. rTgSRS2-based ELISA can differentiate mice serum from *T. gondii* infected and non-infected mice, and there is no cross reactivity with *Neospora caninum*-infected mice sera. To evaluate its usefulness in the field samples, 76 clinical cat samples were collected from Japan and tested for antibodies against *T. gondii*. The results of ELISA had good performance with high sensitivity, specificity and reliable kappa value compared with the results of LAT. This data indicate that the rTgSRS2-based ELISA could be useful tool for epidemiological surveys on toxoplasmosis in the field.

In China, most people like "barbeque" and "slightly boiled mutton"，considering the public health and economic importance of sheep products, a survey on toxoplasmosis in sheep from different part in northern China was done. The TgSRS2-based ELISA and LAT were used to investigate the sero-prevalence of *T. gondii*. LAT is widely used in epidemiological studies of *T. gondii* in many animal species (Matsuo and Husin, 1996, Kyan et al., 2012). The ELISA in this chapter had a high sensitivity, specifity and kappa value relative to LAT. The survey provides a comprehensive record of the seroprevalence of *T. gondii* antibodies in northern China due to the the number of studied areas. Risk factors were analyzed to determine the impact of sampling area, gender, age and rearing system on toxoplasmosis prevalence. There was a significant difference of seroprevalence between sheep from Hebei and Inner Mongolia. This could be due to Inner Mongolia being colder than Hebei. The occysts can hardly develop into an infectious stage under low temperatures. The higher

seropositive rates of cat in Hebei (57.3%) than in Inner Mongolia (10.3%) may also be an explanation (Ding et al., 2017), because cat can significantly affect *T. gondii* transmission (Dubey and Schares, 2011). Animal age is another risk factor that can dramatically influence the seropositivity for *T. gondii* in the investigated areas, which is similar with other reports (Ramzan et al., 2009; Xu et al., 2015), suggesting that toxoplasmosis can be transmitted via horizontal transmission. The sheep raised intensively had higher seropositive rates than those raised extensively, the possible reason is that sheep raised intensively are usually caged leading to fewer chances of infection by oosysts of *T. gondii* excreted in the environment.

General summary

Toxoplasmosis as an important worldwide zoonosis caused by apicomplexan protozoan *T. gondii* and is associated with high morbidity in animals and human beings. Human and animals can be infected by ingestion of contaminated water, vegetables and meat products that contain infectious oocysts or tissue cysts. The increasingly emergence of toxoplasmosis has resulted in demands for effective diagnostic methods and urgent preventive strategies to detect and control the disease.

The main purpose of this study was to develop an accurate diagnostic method against toxoplasmosis. An in-depth understanding of the antigens that play a key role in the growth and virulence of parasites is important in developing a specific and sensitive diagnostic tool. The newly established serological diagnostic method has been applied the detection of specific anti-*T. gondii* antibodies in sheep from northern China.

In chapter 1, functional analysis of TgSRS2 in RH and PLK strains was performed using CRISPR/CAS9 technology. Amino acid sequence alignment showed that TgSRS2 contained four polymorphic amino acids. Invasion assay showed that the percentage of cells infected by ΔTgSRS2 PLK strain was significantly lower than that of those infected by the parental strain. In addition, deletion of TgSRS2 significantly decreased the virulence of the PLK strain in mice. However, no difference was observed between the phenotypes of ΔTgSRS2 RH and parent strains, which could be due to the low expression of TgSRS2 in RH strain. These results indicate that TgSRS2 plays an important role in invasion and virulence of *T. gondii* PLK strain in mice.

In chapter 2, the immunogenicity and antigenicity of rTgSRS2 was evaluated using serum from mice experimentally infected with *T. gondii* and clinical cat samples in an ELISA reaction. The performance of the rTgSRS2 based ELISA was compared with that of LAT. The specificity and sensitivity of the ELISA were 95% and 71%, respectively. ELISA showed high concordance (84%) and substantial agreement (kappa value > 0.61) with the results of LAT. These results suggest that the rTgSRS2-based ELISA could be a useful tool for routine testing in clinical and mass screening of samples in the field.

In chapter 3, a survey was performed to evaluate the seroprevalence of *T. gondii* infection in sheep from northern China and to assess the risk factors for infection using LAT and rTgSRS2-based ELISA. Of the 288 serum samples, 87 samples (30.2%), 101 samples (35.1%), and 73 samples (25.5%) were positive for anti-*T. gondii* antibodies according to LAT, ELISA, and both tests, respectively. Seroprevalence of *T. gondii* infection in sheep was 53.7% in Hebei, 38.8% in Heilongjiang, 29.2% in Jilin and 19.7% in Inner Mongolia using ELISA. Age and rearing system significantly affected seropositivity. The findings obtained in this study may be helpful in designing appropriate measures for the prevention and control of toxoplasmosis in sheep, other animals and humans.

Overall, the present study analyzed the function of SRS2 in *T. gondii*, and evaluated the sensitivity and specificity of rTgSRS2 based ELISA and determined the seroprevalence of *T. gondii* infection in sheep in northern China using this ELISA method. These results provide useful information for understanding the functional characteristic of TgSRS2 and its ability to detect *T. gondii* infection in cats and sheep.

和文要約

トキソプラズマ症は、アピコンプレックス門の原虫 *Toxoplasma gondii* により引き起こされる人獣共通感染症であり、世界的に分布している。人や動物はオーシストに汚染された飲料水や食物ならびにシスト含有食肉を摂取することにより感染する。トキソプラズマ症の流行実態の把握と制御のためには有効な診断法の確立が求められている。

第１章では、トキソプラズマの表面抗原分子である TgSRS2 の性状解析を行った。RH 株（遺伝子 1 型）と PLK 株（遺伝子２型）の TgSRS2 を TgSRS2CRISPR/CAS9 遺伝子編集法によりノックアウト（KO）した後に、この分子の機能解析を行った。両株間には４つのアミノ酸多型が認められた。PLK 株の野生株と KO 株の宿主細胞への侵入率を調べたところ、後者の方が顕著に低下した。また、マウスへの病原性も後者の方が低かった。しかし、RH 株の野生株と KO 株間には宿主細胞への侵入率とマウスへの病原性に有意差が認められなかった。これは、RH 株における TgSRS2 の低発現率に起因すると考えられた。これらの結果により、TgSRS2 は一部の遺伝子型においては虫体の宿主細胞への侵入とマウスへの病原性において重要な役割を果たすことが示唆された。

第２章では、TgSRS2 の抗原性について解析した。大腸菌で発現させた組換え TgSRS2 は、トキソプラズマ実験感染マウス血清と強く反応し、診断用抗原としての有用性が示唆された。次に、トキソプラズマの終宿主である猫

の血清について、組換え TgSRS2 を抗原とした酵素免疫測定（ELISA）法と現行のラテックス凝集（LAT）法の比較検討を行った。ELISA 法は、LAT 法と84%の一致性を示した。また、ELISA 法の特異性と感度はそれぞれ 95%と 71%であった。これらの結果より、組換え TgSRS2 を用いた ELISA 法は猫の血清診断に有用であることが示唆された。

第３章では、組換え TgSRS2 を抗原とした ELISA 法と LAT 法を用いて中国北部地域における羊のトキソプラズマ症に対する血清学調査を行った。羊血清 288 サンプル中、87 サンプル（30.2%）と 101 サンプル（35.1%）がそれぞれ陽性と判定された。また、73 サンプル（25.5%）は両者ともに陽性と判定された。ELISA 法では、河北省、黒竜江省、吉林省、内モンゴル自治区由来の羊血清サンプルの陽性率がそれぞれ 38.8%、28.8%、20.0%、14.5%であった。また、年齢と飼育法による陽性率の変化も認められた。これらの結果より、組換え TgSRS2 を抗原とした ELISA 法は、家畜のトキソプラズマ症の疫学調査に有用であることが示唆された。

以上をまとめると、本研究ではトキソプラズマの表面抗原である TgSRS2 は、虫体の宿主細胞への侵入に関与する分子であり、また、組換え TgSRS2 はトキソプラズマ症の血清診断抗原としても有用であることを示した。

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References

Abdelbaset AE, Alhasan H, Salman D, Karram MH, Ellah Rushdi MA, Xuenan X, Igarashi M. 2017. Evaluation of recombinant antigens in combination and single formula for diagnosis of feline toxoplasmosis. Exp Parasitol. 172, 1-4.

Beghetto E, Buffolan W, Spadoni A, Del Pezzo M, Di Cristina M, Minenkova O, Petersen E, Felici F, and Gargano N. 2003. Use of an immunoglobulin G avidity assay based on recombinant antigens for diagnosis of primary *Toxoplasma gondii* infection during pregnancy. J Clin Microbiol. 41, 5414-5418.

Bhopale GM. 2003. Pathogenesis of toxoplasmosis. Comp Immunol Microbiol Infect Dis. 26, 213–222.

Boothroyd JC. 1993. Population biology of *Toxoplasma*: clonality, virulence, and speciation (or not). Infect Agents Dis. 2, 100-102.

Buxton D. 1998. Protozoan infections (*Toxoplasma gondii, Neospora caninum,* and *Sarcocystis spp.*) in sheep and goats: recent advances. Vet Res. 29, 289-310.

Cai Y, Wang Z, Li J, Li N, Wei F, Liu Q. 2015. Evaluation of an indirect ELISA using recombinant granule antigen Gra7 for serodiagnosis of *Toxoplasma gondii* infection in cats. J Parasitol. 101, 37-40.

Dannemann BR, Vaughan WC, Thullies P, Remington JS. 1990. Differential agglutination test for diagnosis of recently acquired infection with *Toxoplasma gondii*. J Clin Microbiol. 28, 1928-1933.

Ding H, Gao YM, Deng Y, Lamberton PH, Lu DB. 2017. A systematic review and meta-analysis of the seroprevalence of *Toxoplasma gondii* in cats in mainland China. Parasit Vectors. 10, 27.

Donald RGK, Roos DS. 1998. Gene knockouts and allelic replacements in *Toxoplasma gondii*: HXGPRT as a selectable marker for hit-and-run mutagenesis. Mol Biochem Parasitol. 91, 295-305.

Dubey JP. 1986. Toxoplasmosis in cats. Feline Pract. 16, 12-45.

Dubey JP. 1993. *Toxoplasma*, *Neospora*, *Sarcocystis*, and other tissue cyst forming coccidia of humans and animals. In: Kreier JP, editor. Parasitic protozoa, 2nd ed. 6, 1-158.

Dubey JP. 1998. Toxoplasmosis, sarcocystosis, isosporosis, and cyclosporosis. In: Palmer SR, Soulsby EJL, Simpson DJH, editors. Zoonoses. Oxford: Oxford University Press, 579-597.

Dubey JP. 2010. Toxoplasmosis of animals and humans. 2nd ed. CRC Press, 17-231.

Dubey JP. Beattie CP. 1998. Toxoplasmosis of animals and humans. Boca Raton, FL, CRC Press.

Dubey JP, Lindsay DS, Speer CA. 1998. Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. Clin Microbiol Rev. 11, 267-299.

Dubey JP, Miller NL, Frenkel JK. 1970. The *Toxoplasma gondii* oocyst from cat feces. J Exp Med. 132, 636–662.

Dubey JP, Schares G. 2011. Neosporosis in animals-the last five years. Vet Parasitol. 180**,** 90-108.

Dzierszinski F, Mortuaire M, Cesbron-Delauw MF, Tomavo S. 2000. Targeted disruption of the glycosylphosphatidylinositol-anchored surface antigen SAG3 gene in *Toxoplasma gondii* decreases host cell adhesion and drastically reduces virulence in mice. Mol Microbiol. 37, 574-582.

Evans R. 1992. Life cycle and animal infection. In: Ho-Yen DO, Joss AWL, editors. Human toxoplasmosis. Oxford: Oxford University Press, 26-55.

Fereig RM, Kuroda Y, Terkawi MA, Mahmoud ME, Nishikawa Y. 2017. Immunization with *Toxoplasma gondii* peroxiredoxin 1 induces protective immunity against toxoplasmosis in mice. PLoS One. 12, e0176324.

Fischer HG, Stachelhaus S, Sahm M, Meyer HE, Reichman G. 1998. GRA7, an excretory 29 kDa *Toxoplasma gondii* dense granule antigen released by infected host cells. Mol Biochem Parasitol. 91, 251-262.

Flegr J, Prandota J, Sovičková M, Israili ZH. 2014. Toxoplasmosis-a global threat. Correlation of latent toxoplasmosis with specific disease burden in a set of 88 countries. PLoS One. 9, e90203.

Frenkel JK. 1970. Pursuing *toxoplasma*. Journal of Infectious Diseases. 122, 553-559.

Frenkel JK. 2000. Biology of *Toxoplasma gondii*. In: Ambroise-Thomas P, Peterse E, editors. Congenital toxoplasmosis: scientific background, clinical management and control. Paris: Springer-Verlag, 9-25.

Frenkel JK, Dubey JP, Miller NL. 1970. *Toxoplasma gondii* in cats: fecal stages identified as coccidian oocysts. Science. 167, 893-896.

Gavrilescu LC, Denkers EY. 2001. IFN-gamma overproduction and high level apoptosis are associated with high but not low virulence *Toxoplasma gondii* infection. J Immunol. 167, 902-909.

Gebremedhin EZ, Abdurahaman M, Hadush T, Tessema TS. 2014. Seroprevalence and risk factors of *Toxoplasma gondii* infection in sheep and goats slaughtered for human consumption in Central Ethiopia. BMC Res Notes. 7, 696.

Hammond-Aryee K, Van Helden LS, Van Helden PD. 2015. The prevalence of antibodies to *Toxoplasma gondii* in sheep in the Western Cape, South Africa. Onderstepoort J Vet Res. 82, 993.

Hill D, Dubey JP. 2000. *Toxoplasma gondii*: transmission, diagnosis and prevention. Clin Mirobiol Infect. 8, 634-640.

Hill DE, Dubey JP. 2013. *Toxoplasma gondii* prevalence in farm animals in the United States. Int J Parasitol. 43, 107-113.

Hill DE, Dubey JP. 2015. *Toxoplasma gondii*. In: Biology of Foodborne Parasites. CRC Press, 209-222.

Hill DE, Dubey JP. 2016. *Toxoplasma gondii* as a Parasite in Food: Analysis and Control. Microbiol Spectr. 4, doi, 10.1128.

Hosseininejad M. 2012. Evaluation of an indirect ELISA using a tachyzoite surface antigen SAG1 for diagnosis of *Toxoplasma gondii* infection in cats. Exp Parasitol. 132, 556-560.

Huang X, Xuan X, Kimbita EN, Battur B, Miyazawa T, Fukumoto S, Mishima M, Makala LH, Suzuki H, Sugimoto C, Nagasawa H, Fujisaki K, Mikami T, Igarashi I. 2002. Development and evaluation of an enzyme-linked

immunosorbent assay with recombinant SAG2 for diagnosis of *Toxoplasma gondii* infection in cats. J Parasitol. 88, 804-807.

Jackson MH, Hutchison WM. 1989. The prevalence and source of *Toxoplasma* infection in the environment. Adv Parasitol. 28, 55-105.

Jacobs D, Vercammen M, Saman E. 1999. Evaluation of recombinant dense granule antigen 7 (GRA7) of *Toxoplasma gondii* for detection of immunoglobulin G antibodies and analysis of a major antigenic domain. Clin Diagn Lab Immunol. 6, 24-29.

Jones J, Lopez A, Wilson M. 2003. Congenital toxoplasmosis. Am Fam Physician. 67, 2131-2138.

Jones JL, Dubey JP. 2012. Foodborne toxoplasmosis. Clin Infect Dis. 55, 845-851.

Jung C, Lee CY, Grigg ME. 2004. The SRS superfamily of *Toxoplasma* surface proteins. Int J Parasitol. 34, 285-296.

Kim SK, Karasov A, Boothroyd JC. 2007. Bradyzoite-specific surface antigen SRS9 plays a role in maintaining *Toxoplasma gondii* persistence in the brain and in host control of parasite replication in the intestine. Infect Immun. 75, 1626-1634.

Kimbita EN, Xuan X, Huang X, Miyazawa T, Fukumoto S, Mishima M, Suzuki H, Sugimoto C, Nagasawa H, Fujisaki K, Suzuki N, Mikami T, Igarashi I. 2001. Serodiagnosis of *Toxoplasma gondii* infection in cats by enzyme-linked immunosorbent assay using recombinant SAG1. Vet Parasitol. 102, 35-44.

Khanaliha K, Motazedian MH, Kazemi B, Shahriari B, Bandehpour M, Sharifniya Z. 2014. Evaluation of recombinant SAG1, SAG2, and SAG3 antigens for serodiagnosis of toxoplasmosis. Korean J Parasitol. 52, 137-142.

Kotresha D, Noordin R. 2010. Recombinant proteins in the diagnosis of toxoplasmosis. APMIS. 118, 529-542.

Kyan H, Taira M, Yamamoto A, Inaba C and Zakimi S. 2012. Isolation and characterization of *Toxoplasma gondii* genotypes from goats at an abattoir in Okinawa. Jpn J Infect Dis. 65, 167-170.

Lappin MR, Powell CC. 1991. Comparison of latex agglutination, indirect hemagglutination, and ELISA techniques for the detection of *Toxoplasma gondii*-specific antibodies in the serum of cats. J Vet Intern Med. 5, 299-301.

Leesombun A, Boonmasawai S, Shimoda N, Nishikawa Y. 2016. Effects of extracts from Thai Piperaceae plants against infection with *Toxoplasma gondii*. PLoS One. 11, e0156116.

Lekutis C, Ferguson DJ, Grigg ME, Camps M, Boothroyd JC. 2001. Surface antigens of *Toxoplasma gondii*: variations on a theme. Int J Parasitol. 31, 1285-1292.

Levine ND. 1961. Protozoan Parasites of domestic animals and humans. Chapter 12, Sarcocystis, *Toxoplasma* and related protozoa. Minneapolis: Burgess Publishing Company, 317-346.

Literák I, Rychlík I, Svobodová V, Pospíšil Z. 1998. Restriction fragment length polymorphism and virulence of Czech *Toxoplasma gondii* strains. Int J Parasitol. 28, 1367-1374.

Liu ZK, Li JY, Pan H. 2015. Seroprevalence and risk factors of *Toxoplasma gondii* and *Neospora caninum* infections in small ruminants in China. Prev Vet Med. 118, 488-492.

Manger ID, Hehl AB, Boothroyd JC. 1998. The surface of *Toxoplasma* tachyzoites is dominated by a family of glycosylphosphatidylinositolanchored antigens related to SAG1. Infect Immun. 66, 2237-2244.

Manger ID, Hehl AB, Boothroyd JC. 1908. The Surface of *Toxoplasma* Tachyzoites is dominated by a family of glycosylphosphatidylinositol-anchored antigens related to SAG1. Infect Immun. 66, 2237-2244.

Marcolino PT, Silva DA, Leser PG, Camargo ME, Mineo JR. 2000. Molecular markers in acute and chronic phases of human toxoplasmosis: determination of immunoglobulin G avidity by Western blotting. Clin Diagn Lab Immunol. 7, 384-389.

Matsuo K, Kamai R, Uetsu H, Goto H, Takashima Y, Nagamune K. 2014. Seroprevalence of *Toxoplasma gondii* infection in cattle, horses, pigs and chickens in Japan. Parasitol Int. 63, 638-639.

Mineo JR, McLeod R, Mack D, Smith J, Khan IA, Ely KH, Kasper LH. 1993. Antibodies to *Toxoplasma gondii* major surface protein (SAG-1, P30) inhibit infection of host cells and are produced in murine intestine after peroral infection. J Immunol. 150, 3951-3964.

Montoya JG. 2002. Laboratory diagnosis of *Toxoplasma gondii* infection and toxoplasmosis. J Infect Dis. 185, S73-S82.

Montoya J, Liesenfeld O. 2004. Toxoplasmosis. Lancet. 363, 1965-1976.

Mordue DG, Monroy F, La Regina M, Dinarello CA, Sibley LD. 2001. Acute toxoplasmosis leads to lethal overproduction of Th1 cytokines. J Immunol. 167, 4574-4584.

Morrissette NS, Sibley LD. 2002. Cytoskeleton of Apicomplexan parasites. Microbiol Mol Biol Rev. 66, 21-38.

Nicolle C, Manceaux L. 1908. Sur une infection a corps de Leishman (ou organismes voisins) du gondi. C R Acad Sci. 147, 736-765.

Nigro M, Gutierrez A, Hoffer AM, Clemente M, Kaufer F, Carral L, Martin V, Guarnera EA, Angel SO. 2003. Evaluation of *Toxoplasma gondii* recombinant proteins for the diagnosis of recently acquired toxoplasmosis by an immunoglobulin G analysis. Diagn Micr Infec Dis. 47, 609-613.

Özmutlu ÇD, Karatepe B. 2017. Seroprevalence of *Toxoplasma gondii* in sheep from Nevşehir Province in Turkey. Terkiye Parazitol Derg. 41, 148-151.

Peixoto L, Chen F, Harb OS, Davis PH, Beiting DP, Brownback CS, Ouloguem D, Roos DS. 2010. Integrative genomic approaches highlight a family of parasite-specific kinases that regulate host responses. Cell Host Microbe. 8, 208-218.

Pfrepper KI, Enders G, Gohl M, Krczal D, Hlobil H, Wassenberg D, Soutschek E. 2005. Seroreactivity to and avidity for recombinant antigens in toxoplasmosis. Clin Diagn Lab Immunol. 12, 977-982.

Pietkiewicz H, Hiszczyńska-Sawicka E, Kur J, Petersen E, Nielsen HV, Stankiewicz M, Andrzejewska I, Myjak P. 2004. Usefulness of *Toxoplasma gondii*-specific recombinant antigens in serodiagnosis of human toxoplasmosis. J Clin Microbiol. 42, 1779-1781.

Qiu JH, Wang CR, Zhang X, Sheng ZH, Chang QC, Zhao Q, Wu SM, Zou FC, Zhu XQ. 2012., Seroprevalence of *Toxoplasma gondii* in beef cattle and dairy cattle in northeast China. Foodborne Pathog Dis. 9, 579-582.

Rachinel N, Buzoni-Gatel D, Dutta C, Mennechet FJ, Luangsay S, Minns LA, Grigg ME, Tomavo S, Boothroyd JC, Kasper LH. 2004. The induction of acute ileitis by a single microbial antigen of *Toxoplasma gondii*. J Immunol. 173, 2725-2735.

Rahman M, Azad MT, Nahar L, Rouf SM, Ohya K, Chiou SP, Baba M, Kitou K, Takashima Y. 2014. Age-specificity of *Toxoplasma gondii* seroprevalence in sheep, goats and cattle on subsistence farms in Bangladesh. J Vet Med Sci. 76, 1257-1259.

Ramzan M, Akhtar M, Muhammad F, Hussain I, Hiszczyńska-Sawicka E, Haq AU, Mahmood MS, Hafeez MA. 2009. Seroprevalence of *Toxoplasma gondii* in sheep and goats in Rahim Yar Khan (Punjab), Pakistan. Trop Anim Health Prod. 41, 1225-1229.

Remington JS, Desmonts G. 1990. Toxoplasmosis. In: Remington JS, Klein JO, editors. Infectious diseases of the fetus and newborn infant, 3rd ed. Philadelphia: WB Saunders, 89-195.

Remington JS, Mcleod R, Desmonts G. 1995. Toxoplasmosis. In: Remington JS, Klein JO, eds. Infectious disease of the fetus and newborn infant. Philadelphia: W.B. Saunders Company, 140-267.

Reynolds MG, Oh J, Roos DS. 2001. In vitro generation of novel pyrimethamine resistance mutations in the *Toxoplasma gondii* dihydrofolate reductase. Antimicrob Agents Chemother. 45, 1271-1277.

Robert-Gangneux F, Daede ML. 2012. Epidemiology of and diagnostic strategies for toxoplasmosis. Clin Microbiol Rev. 25, 264-296.

Saeij JP, Boyle JP, Coller S, Taylor S, Sibley LD, Brooke-Powell ET, Ajioka JW, Boothroyd JC. 2006. Polymorphic secreted kinases are key virulence factors in toxoplasmosis. Science. 314, 1780-1783.

Saeij JP, Coller S, Boyle JP, Jerome ME, White MW, Boothroyd JC. 2007. *Toxoplasma* co-opts host gene expression by injection of a polymorphic kinase homologue. Nature. 445, 324-327.

Scherf A, Hernandez-Rivas R, Buffet P, Bottius E, Benatar C, Pouvelle B, Gysin J, Lanzer M. 1998. Antigenic variation in malaria: in situ switching, relaxed and mutually exclusive transcription of var genes during intraerythrocytic development in Plasmodium falciparum. EMBO J. 17, 5418-5426.

Shahiduzzaman M, Islam R, Khatun MM, Batanova TA, Kitoh K, Takashima Y. 2011. *Toxoplasma gondii* seroprevalence in domestic animals and humans in Mymensingh District, Bangladesh. J Vet Med Sci. 73, 1375-1376.

Shen B, Brown KM, Lee TD, Sibley LD. 2014. Efficient gene disruption in diverse strains of *Toxoplasma gondii* using CRISPR/CAS9. MBio. 5, e01114.

Shiadeh MN, Moghadam ZB, Adam I, Saber V, Bagheri M, Rostami A. 2017. Human infectious diseases and risk of preeclampsia: an updated review of the literature. Infection. 45, 589-600.

Singh H, Tewari AK, Mishra AK, Maharana B, Sudan V, Raina OK, Rao JR. 2015. Detection of antibodies to *Toxoplasma gondii* in domesticated ruminants by recombinant truncated SAG2 enzyme-linked immunosorbent assay. Trop Anim Health Prod. 47, 171-178.

Tegegne D, Kelifa A, Abdurahaman M, Yohannes M. 2016. Seroepidemiology and associated risk factors of *Toxoplasma gondii* in sheep and goats in Southwestern Ethiopia. BMC Vet Res. 12, 280.

Templeton TJ. 2007. Whole-genome natural histories of apicomplexan surface proteins. Trends Parasitol. 23, 205-212.

Tenter AM, Heckeroth AR, Weiss LM. 2000. *Toxoplasma gondii*: from animal to humans. Int J Parasitol. 30, 1217-1258.

Terkawi MA, Kameyama K, Rasul NH, Xuan X, Nishikawa Y. 2013. Development of an immunochromatographic assay based on dense granule protein 7 for serological detection of *Toxoplasma gondii* infection. Clin Vaccine Immunol. 20, 596-601.

Tomavo S, Schwarz RT, Dubremetz JF. 1989. Evidence for glycosylphosphatidylinositol anchoring of *Toxoplasma gondii* major surface antigens. Mol Cell Biol. 9, 4576-4580.

Villavedra M, Battistoni J, Nieto A. 1999. IgG recognizing 21–24 kDa and 30–33 kDa tachyzoite antigens show maximum avidity maturation during natural and accidental human toxoplasmosis. Rev Inst Med Trop Sao Paulo. 41, 297-303.

Wang CR, Qiu JH, Gao JF, Liu LM, Wang C, Liu Q, Yan C, Zhu XQ. 2011. Seroprevalence of *Toxoplasma gondii* infection in sheep and goats in northeastern China. Small Rumin Res. 97, 130-133.

Wasmuth J, Daub J, Peregrín-Alvarez JM, Finney CA, Parkinson J. 2009. The origins of apicomplexan sequence innovation. Genome Res. 19, 1202-1213.

Wasmuth JD, Pszenny V, Haile S, Jansen EM, Gast AT, Sher A, Boyle JP, Boulanger MJ, Parkinson J, Grigg ME. 2012. Integrated bioinformatic and targeted deletion analyses of the SRS gene superfamily identify SRS29C as a negative regulator of Toxoplasma virulence. MBio. 3, e00321.

Webster JP. 2007. The Effect of *Toxoplasma gondii* on Animal Behavior: Playing Cat and Mouse. Schizophr Bull. 33, 752-756.

Weiss LM, Udem SA, Tanowitz HB, Wittner M. 1998. Western blot analysis of the antibody response of patients with AIDS and *toxoplasma* encephalitis: antigenic diversity among *Toxoplasma* strains. J Infect Dis. 157, 7-13.

Wu SM, Danba C, Huang SY, Zhang DL, Chen J, Gong G, Xu MJ, Yuan ZG, Zhu XQ. 2011. Seroprevalence of *Toxoplasma gondii* infection in Tibetan sheep in Tibet, China. J Parasitol. 97, 1188-1189.

Xu P, Li X, Tang F, Liu YH, Kou X, Zhao ML, Li B, Guo L, Liu XG, Zhao Q. 2015. Seroprevalence and risk factors for *Toxoplasma gondii* in sheep and goats in Jinzhou, Northeastern China. Trop Biomed. 32, 563-567.

Yang N, Li H, He J, Mu M, Yang S. 2013. Seroprevalence of *Toxoplasma gondii* infection in domestic sheep in Liaoning Province, northeastern China. J Parasitol. 99, 174-175.

Yang M, Zheng J, Jia H, Song M. 2016. Functional characterization of X-prolyl aminopeptidase from *Toxoplasma gondii*. Parasitology. 143, 1443-1449.

Yin MY, Wang JL, Huang SY, Qin SY, Zhou DH, Liu GX, Tan QD, Zhu XQ. 2015. Seroprevalence and risk factors of *Toxoplasma gondii* in Tibetan Sheep in Gansu province, Northwestern China. BMC Vet Res. 11, 41.

Zhang N, Wang S, Wang D, Li C, Zhang Z, Yao Z, Li T, Xie Q, Liu S, Zhang H. 2016. Seroprevalence of *Toxoplasma gondii* infection and risk factors in domestic sheep in Henan province, central China. Parasite. 23, 53.

Zheng J, Jia H, Zheng Y. 2015. Knockout of leucine aminopeptidase in *Toxoplasma gondii* using CRISPR/Cas9. Int J Parasitol. 45, 141-148.

Zheng J, Cheng Z, Jia H, Zheng Y. 2016. Characterization of aspartyl aminopeptidase from *Toxoplasma gondii*. Sci Rep. 6, 34448.

Zou F, Yu X, Yang Y, Hu S, Chang H, Yang J, Duan G. 2015. Seroprevalence and risk factors of *Toxoplasma gondii* infection in buffaloes, sheep and goats in Yunnan Province, Southwestern China. Iran J Parasitol. 10, 648-651.