Native SAG1 in *Toxoplasma gondii* lysates is superior to recombinant SAG1 for serodiagnosis of *T. gondii* infections in chickens

Cornelia Appiah-Kwarteng[a,b], Taizo Saito[a,b], Natsuki Toda[b], Katsuya Kitoh[a,b], Yoshibumi Nishikawa[c], Christopher Adenyo[d], Boniface Kayang[d,e], Ebenezer Oduro Owusu[f], Kenji Ohya[a,g], Miho Inoue-Murayama[h], Fumiya Kawahara[i,j], Kisaburo Nagamune[i,k], Yasuhiro Takashima[a,b,l,*]

[a] *The United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan*
[b] *Department of Veterinary Parasitological Diseases, Faculty of Applied Biological Science, 1–1 Yanagido, Gifu 501-1193, Japan*
[c] *National Research Center for Protozoan Diseases (NRCPD), Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido, Japan*
[d] *Livestock and Poultry Research Centre, College of Basic and Applied Sciences, University of Ghana, P.O. Box LG 25, Legon, Accra, Ghana*
[e] *Department of Animal Science, College of Basic and Applied Sciences, University of Ghana, P.O. Box LG 25, Legon, Accra, Ghana*
[f] *Department of Animal Biology and Conservation Science, University of Ghana, P.O. Box LG 25, Legon, Accra, Ghana*
[g] *Department of Microbiology, Faculty of Applied Biological Science, 1–1 Yanagido, Gifu 501-1193, Japan*
[h] *Wildlife Research Center of Kyoto University, 2-24 Tanaka-Sekiden-cho, Kyoto 606-8203, Japan*
[i] *Department of Parasitology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan*
[j] *Mocky Poultry Practice, Shinmeidai 2-5-33-810, Hamura, Tokyo 205-0023, Japan*
[k] *Graduate School of Life and Environmental Sciences, University of Tsukuba, Tennodai 1-1-1, Tsukuba, Ibaraki 305-8572, Japan*
[l] *Center for Highly Advanced Integration of Nano and Life Sciences, Gifu University (G-CHAIN), 1-1 Yanagido, Gifu 501-1193, Japan*

ARTICLE INFO

ABSTRACT

Toxoplasma gondii can infect almost all mammals and birds, including chickens. The aim of this study was to identify an appropriate immunogenic antigen for serodiagnosis of *T. gondii* infections in chickens. We examined serum samples from chickens that were intravenously or intraperitoneally infected with 10^6–10^8 tachyzoites of *T. gondii* strains PLK, RH, CTG, ME49 or TgCatJpGi1/TaJ using enzyme-linked immunosorbent assays (ELISAs), latex agglutination tests (LATs) and western blotting. Regardless of parasite strain or infection dose and route, the commercial LAT was positive for almost all sera collected 1 week post-infection. However, at 2 weeks post-infection, LATs were negative in the same birds. ELISAs using the *Escherichia coli*-produced recombinant *T. gondii* antigens SAG1 and GRA7 showed strong signals at 1–2 weeks post infection, but thereafter diminished for the majority of serum samples. In contrast, western blotting against crude tachyzoite antigens showed a persistent band up to 4 weeks post-infection. Sera from these chickens reacted much more strongly with SAG1 from crude tachyzoite antigens than with recombinant SAG1. Even in experimentally-infected birds whose parasite burdens in tissue were undetectable, sera still reacted with native SAG1. We tested sera from free-range chickens on a small farm in Ghana, Africa, using western blotting and found that the serum of one bird reacted with a single band of approximately 27 kDa, the putative molecular weight of SAG1. Thus we conclude that native SAG1, but not *E. coli*-produced recombinant SAG1, is suitable for serodiagnosis of *T. gondii* infections in chickens.

1. Introduction

Toxoplasma gondii, a zoonotic protozoan parasite, causes widespread infections in humans and other animals, including chickens. [1]. Although most infections in humans are asymptomatic, the parasite can cause severe complications in immunocompromised individuals, and abortion when a mother is infected for the first time during pregnancy [2]. Human toxoplasmosis is transmitted by ingesting the tissue cysts present in infected raw or undercooked meat products derived from domestic animals latently infected with *T. gondii*, or through oral contact with soil or water contaminated with oocysts in the feces from infected cats. Surveillance of soil and/or water for contamination with *T. gondii* oocysts is required to evaluate the risk of acquiring *T. gondii* infections in humans. However, the lack of sensitive methods means

---

that oocysts are rarely recovered in field investigations. Therefore, instead of using a method that can directly detect oocysts, it has been proposed that the seroprevalence of *T. gondii* in animals in environmentally-susceptible areas is surveyed. Chickens have specifically been used for this purpose because they feed directly from the ground and are therefore likely to come into contact with parasite-contaminated materials [3,4]. But for this approach to be successful, it is essential to accurately establish which sentinel animals have been exposed to *T. gondii*.

Recombinant proteins, which are easily produced on a large scale, can be good diagnosis antigens for the serodiagnosis of sentinel animals. Recombinant proteins produced in *Escherichia coli* are widely used to detect anti-*T. gondii* antibodies in humans and domestic animals [5–7]. However, when E *coli*-derived recombinant GRA1, GRA6, GRA9 and SAG1 were used as diagnostic antigens, anti-*T. gondii* antibodies in experimentally infected chickens were detected for only a short period, and the detected antibody titers decreased almost immediately to undetectable levels [8]. Therefore, current serodiagnosis systems using recombinant antigens only detect recent infections and miss individuals that were infected a while ago. Therefore, the aim of this study is to identify parasite proteins that are suitable antigens for detecting both old and new *T. gondii* infections in chickens.

In this study, we found that sera from experimentally infected chickens had long-lived reactions with parasite-derived authentic SAG1 but not with *E. coli*-derived recombinant SAG1.

2. Materials and methods

2.1. Construction of a recombinant T. gondii parasite expressing a red fluorescent protein

2.1.1. TgCatJpGi1/TaJ/GRA red

The DHFR-TSc3 pyrimethamine resistance gene was PCR amplified from the pDHFR-TSc3 plasmid [9] using the following primers: DHFR-F: 5′-CACTCATTAGGCACCCCAGGCTTTACACTTT-3′ and DHFR-R: 5′-GGTGGCGGCCGCTCTAGAACTAGTGGATCG-3′. A 3′ Terminal A was added to the amplified fragment using the 10 × A-attachment kit (Toyobo, Japan), and the fragment was inserted into the pGEM-T easy vector (Promega, USA) by TA cloning; this plasmid is hereafter called pGEM-DHFR. GRA1pro and GRA2ter genes were PCR amplified from the pDMG plasmid according to the method used in Nishikawa et al., 2003 [10] Primers containing *Xba*1 (Gra1pro F: 5′Gra1pTCTAGAGAA GGCTGTAGTACTGGTG-3′) and *Nco*1 (Gra2ter R: 5′Gra2tCCATGGGTC GACTGGAACTACGGTG-3′) restriction enzyme sites (underlined) were designed. The PCR-amplified fragment from these primers was ligated to the *Xba1/Nco*1 site in pGEM-DHFR, and the resultant plasmid is hereafter called pDHFR-GG. The DNA fragment encoding the red fluorescent protein DsRed Express (Takara, Japan) was inserted into the *EcoRV* site of pDHFR-GG, and the resultant plasmid is hereafter called pToxo-DsRed-DHFR. pToxo-DsRed-DHFR (200 μg) was electroporated into TgCatJpGi1/TaJ tachyzoites [11] using Gene Pulser (Bio-Rad, USA), and the transfected parasites were transferred in electroporation medium (1 M KCl, 100 mM CaCl$_2$.2H$_2$O, 1 M K$_2$HPO$_4$/KH$_2$PO$_4$, pH 7.6; 1 M HEPES, pH 7.6; 100 mM EDTA, 1 M MgCl$_2$.6H$_2$O) to a Vero cell culture. Forty-eight h after electroporation, the culture medium was changed to RPMI 1640 medium containing 1 μM pyrimethamine, 7.5% fetal calf serum and 20 μg/ml gentamicin. After antibiotic selection, the culture was subjected to limiting dilution and the resulting fluorescent clones were designated TgCatJpGi1/TaJ/GRA Red.

2.2. Parasite preparation

T. gondii parasite strains RH, CTG, ME49, PLK and TgCatJpGi1/TaJ/GRA Red were maintained in Vero cell cultures. To collect the parasites, the Vero cells were scraped from the inside of the culture flasks and each preparation was passed through an unused 27^3/$_4$ G needle, three

times. The cells were pelleted at 2000 rpm for 10 min, washed in PBS, and then passed through an unused 5 μm filter (Millipore Corporation, USA). Parasite numbers were determined using a cell counter and a microscope (Olympus CKX41, Olympus Corporation, Japan). Parasites were then repelleted at 2000 rpm for 10 min.

2.3. Infections in chickens

The collected parasites were suspended in the amount of PBS appropriate to produce the required number of tachyzoites in 100 μl, and then injected into 1-month-old White Leghorn chickens or hybrid chickens for egg production. For the latex agglutination test, White leghorn chickens were intravenously infected with a 100 μl solution containing 1×10^7 tachyzoites of the RH or the CTG strain ($n = 3$). White leghorn chickens were also intravenously injected with 1×10^6, 1×10^7 and 1×10^8 Me49 tachyzoites (2 chickens/dose). Individual chickens were also infected with 1×10^6, 1×10^7 or 1×10^8 parasites, intraperitoneally. Hybrid chickens were intravenously injected with 1×10^7 PLK tachyzoites. For the enzyme-linked immunosorbent assays (ELISAs), three White Leghorn chickens were intravenously injected with 1×10^7 RH tachyzoites, and two, one and four hybrid chickens were intravenously injected with 1×10^5, 1×10^6 and 1×10^7 PLK tachyzoites, respectively. For the western blot analysis, the chickens were intravenously infected with 1×10^7 PLK tachyzoites. Blood was collected from the wing vein once a week and serum samples were stored at –20 °C until further analysis.

To examine the parasite burden in the chicken tissues, seven day-old hybrid chicks were intraperitoneally infected with 1×10^7 TgCatJpGi1/TaJ/GRA Red tachyzoites. All chickens were housed at the BSL2 poultry house in Gifu University. All the experiments using chickens were performed in accordance with the Gifu University Animal Care and Use guidelines from the Committee (Permission No. 17061).

2.4. Latex agglutination test

The Toxocheck-MT kit (Eiken Kagaku, Japan) was used in this experiment. Serum samples were diluted 16, 32 and 64 times and scoring was determined as per the manufacturer's instructions. Samples were recorded as '+' when the 32 times dilution produced a score of more than 1. '±' was recorded when samples that were diluted 16 times produced a score of 1. Samples denoted '–' produced a score of less than 1 in the 16 times diluted solutions.

2.5. ELISAs

Using GRA7 and SAG1 as the antigens, ELISAs were performed using each infected chicken's serum sample. GST-GRA7 or GST-SAG1 [12,13] (50 μl of a 0.1 μM concentration) was loaded onto a 96-well plate (Costar 9018 EIA/RIA plate 96-well flat bottomed, ImmunoChemistry Technologies, LLC, Bloomington, MN, USA). The plate was incubated at 4 °C overnight, and washed once with 0.05% PBS-Tween® 20 (PBS-T). Skimmed milk (3%, 100 μl/well) was added and the plate was incubated at 37 °C for 1 h, and then washed once with 200 μl/well of PBS-T. Primary antibody (50 μl/well of a 1:100 dilution of infected chicken serum in 3% skimmed milk) was added and the plate incubated at 37 °C for 1 h, and then washed six times with 200 μl/well of PBS-T. The secondary antibody (50 μl of a 1:4000 dilution of goat-anti chicken IgY (Santa CruzBiotechnology, Santa Cruz, CA, USA) in 3% skimmed milk) was added per well and the plate was again incubated at 37 °C for 1 h. After rinsing six times with 200 μl/well of PBS-T, 100 μl/well of substrate (0.05% ABTS; 0.1 M citric acid, 0.2 M buffer, pH 4.0; 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); 0.003% hydrogen peroxide) was added, the plate was incubated at room temperature for 1 h, and then read using a microplate reader (Model 680 Microplate reader, Bio-Rad, Hercules, CA, USA) at 415 nm.

2.6. Western blot analysis

Purified parasites were lysed with M-PER™ Mammalian Protein Extraction Reagent (Thermo Scientific, Waltham, MA, USA) and an equal volume of Electrophoresis Sample Buffer (Santa Cruz Biotech) was added to the preparation, which was then heated at 95 °C for 5 min. The crude antigen (15 μl) was loaded and the proteins within it separated by 5–20% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Antigen transfer from the gel to a nitrocellulose membrane was done using Trans-blot SD semi-dry Transfer Cell apparatus (Bio-Rad), and the membrane was incubated in 3% skimmed milk overnight. After incubation with the infected chicken serum (1:1000 dilution in 3% skimmed milk) at 37 °C for 1 h, the membrane was given six 5 min washes in PBS containing Tween® 20 (Takara Bio, Shiga, Japan). The individual membranes were again incubated in goat-anti chicken IgY-HRP (Santa Cruz Biotechnology) (1:20000 dilution in 3% skimmed milk) at 37 °C for 1 h, and then washed in PBS-T six times for 5 min each time. Chemiluminescent images were developed using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, CHI, Illinois, USA) and film (Fuji Medical X-ray film, Fujifilm, Tokyo). The Ghanaian field samples were analyzed using 1-Step™ TMB-Blotting Substrate Solution (Thermo Scientific) to visualize the reactive bands according to the manufacturer's instructions instead of the Amersham ECL Prime Western Blotting Detection Reagent. To compare the immunogenicities of the parasite-derived authentic SAG1 protein and the *E.coli* derived recombinant SAG1, crude tachyzoite antigen was serially diluted and 3.0 μg, 1.5 μg, 0.75 μg, 0.38 μg and 0.19 μg of the purified recombinant SAG1 protein was electrophoretically separated by SDS-PAGE as described above. To visualize and compare the amount of SAG1 in the lanes, the gel was stained using 2D-Silver Stain Reagent II (Cosmo Bio, Japan) according to the manufacturer's instructions. The crude tachyzoite antigen and purified recombinant SAG1 samples containing comparable amount of SAG1 protein were used for western blot analysis, as described above. To examine the serum from the mice that had been used for the bioassays, western blot analysis using crude PLK tachyzoite antigen and mouse serum, each at a dilution of 1:1000, was used. Rabbit anti mouse secondary antibody (Thermofisher, Waltham, MA, USA) was then used at a dilution of 1:20000. Visualization was done by chemiluminescence.

2.7. Antigen identification

The crude tachyzoite antigen, as prepared above, was run on several lanes of a 5–20% SDS gel and the pre-identified immunogenic band was excised. The amino acid sequences of the digested immunogenic proteins were identified by liquid chromatography-mass spectrometry. BLAST analysis (National Center for Biotechnology Information, http://blast.ncbi.nlm.nih.gov/) was used to identify the amino acid sequences matching those of the peptides identified in the samples.

2.8. Observing fluorescent parasites in tissue samples from chickens

The heart, brain, liver and lungs from each animal were examined in the chickens previously injected with TgCatJpGi1/TaJ/GRA Red tachyzoites at 6 weeks post infection. To determine whether infectious parasites existed in these tissues, the tissue samples were observed for the presence of fluorescent parasites. Half of each heart, brain, liver and lungs were cut into small pieces, crushed between two glass slides and then observed by microscopy.

2.9. Bioassays

The remaining halves of the hearts, brains, livers and lungs from TgCatJpGi1/TaJ/GRA Red tachyzoite-injected chickens at 6 weeks post infection were also examined for the presence of parasites using a mouse bioassay, as described in Dubey, 1998 [14]. Briefly, half of each tissue sample was homogenized and intraperitoneally injected into two C57BL/6 mice per sample. The mice were observed for the clinical signs of toxoplasmosis, and after 4 weeks their serum samples were tested for antibodies using western blot analysis. All the experiments using these mice were performed in accordance with the Gifu University Animal Care and Use guidelines from the Committee (Permission No. 17060).

2.10. Field sampling

Sampling was done on two farms in Ghana, West Africa. The first was The Livestock and Poultry Research Center (LIPREC) at the University of Ghana, located in Accra, Ghana's capital city, where 10 chickens were sampled from a caged house. The second was a local farm located in Nsawam, about 30 km from the capital city, where 10 free-range chickens were sampled. Blood was collected from the wing veins of the chickens.

3. Results

3.1. Latex agglutination detection of chicken anti-T. gondii antibodies

To investigate whether a serodiagnosis system authorized for use as an inspection agent for animal species other than chickens could also be used in chickens, we tested serum samples from experimentally infected chickens with Toxocheck-MT (Eiken Kagaku, Japan). Toxocheck-MT, a latex agglutination-type test, has been authorized as an inspection agent for cats and pigs, but not for chickens. However, this kit has been widely used in field surveys to obtain a general picture of the prevalence of *T. gondii* infections in chickens.

Chickens were infected with different strains and doses of *T. gondii* parasites, as described in Table 1. No chickens showed the clinical signs of toxoplasmosis. With the exception of a few individuals (chickens 13, 15, 16, 19 and 20) which never showed positive reactions during the examination period, the majority of individuals showed either positive or quasi-positive reactions at 7 days post infection. However, all

Table 1
Latex agglutination test on sera from infected chickens.

Chicken type	ID	Strain	Infection route	Dose	Days post infection				
					0	7	14	21	28
White leghorn (SPF)	1	RH	i.v.	1×10^7	−	+	−	−	−
	2				−	+	−	−	−
	3				−	+	−	−	−
	4	CTG	i.v.	1×10^7	−	+	−	−	−
	5				−	+	−	−	−
	6				−	±	−	−	−
	7	ME49	i.v.	1×10^6	n.d	+	−	−	−
	8				n.d	+	−	−	−
	9			1×10^7	n.d	+	−	−	−
	10				n.d	±	−	−	−
	11			1×10^8	n.d	+	−	−	−
	12				n.d	+	−	−	−
	13		i.p.	1×10^6	n.d	−	−	−	−
	14			1×10^7	n.d	±	−	−	−
	15			1×10^8	n.d	−	−	−	−
Hybrid for eggs	16	PLK	i.v.	1×10^7	−	−	−	−	−
	17				−	±	−	−	−
	18				−	±	−	−	−
	19				−	−	−	−	−
	20				−	−	−	−	−
	21				−	+	−	−	−

Sera were diluted 16, 32 and 64 fold. Scoring was done as per the manufacturer's instruction as follows:
+ , 0.32 times diluted serum with a score of 1.
± , 0.16 times diluted serum with a score of 1.
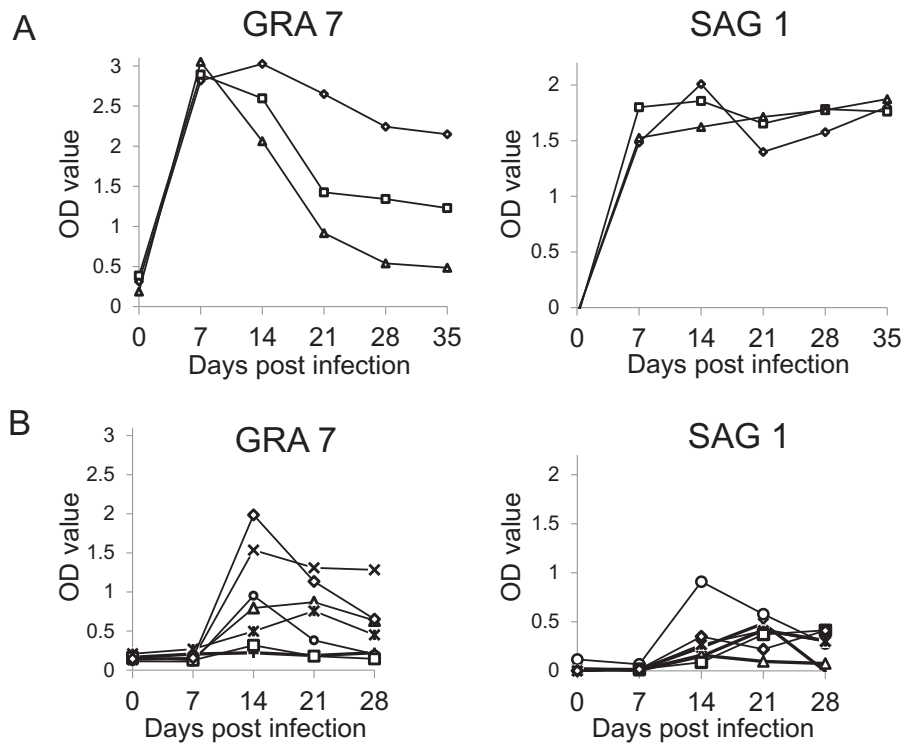− , 0.16 times diluted serum with a score of less than 1.
n.d, not done.

Fig. 1. ELISA using *E.coli*-produced GRA 7 and SAG1 proteins. ELISAs were performed using GRA 7 and SAG1 as the antigens and the sera from chickens infected with A) 1×10^7 *T. gondii* RH strain tachyzoites and B) 1×10^5–1×10^7 PLK strain tachyzoites. Infection was initiated by the intravenous route in all birds.

individuals, including those that had shown a clear positive reaction at 7 days post infection, did not show a positive reaction after 14 days post infection. Although the latex aggregation test does not require species-specific secondary antibodies and therefore has the potential to be used across animal species, no chickens tested positive for *T. gondii* from 14 days post infection using this system.

3.2. ELISA-based detection of chicken anti-T. gondii antibodies using E. coli-produced antigens

Sera from the experimentally infected chickens were examined by ELISA using recombinant *E. coli*-produced GRA7 and SAG1 as antigens. Anti-SAG1 antibodies in the RH strain-infected chickens were stably detected by the ELISA across all the examination periods (Fig. 1A). However, the anti-GRA7 antibody titers detected by the ELISA system peaked at 7–14 days post infection, decreasing soon afterwards (Fig. 1A). In the case of the PLK strain-infected chickens, the titers of both anti-SAG1 and anti-GRA7 antibodies peaked at 7–14 days post infection but the titers for the majority of individuals decreased immediately afterwards (Fig. 1B). Five serum samples previously used in the latex aggregation test (Table 1) were also analyzed by ELISA. These sera also tested positive at 7 days post infection, but the antibody titers for some individuals decreased immediately thereafter (Supplementary Fig. 1).

3.3. Search for the T. gondii antigens recognized continuously by IgY antibodies in T. gondii infected chickens

To identify antigens continuously recognized by IgY in the *T. gondii* infected chickens, the reactions of serum samples obtained from these chickens (ID numbers 4, 5 and 6 in Table 1) with crude tachyzoite lysate were examined by western blot analysis. As shown in Fig. 2A, on day 7 post infection, several bands with a variety of molecular weights reacted with the serum (Fig. 2A). Although some bands began to disappear after the second week, a few bands remained and persisted

throughout the observation period (Fig. 2A). We identified one of these persistent bands as SAG1 using LC-MS (Fig. 2B). The SAG1 reaction was consistent irrespective of the *T. gondii* strain used to infect the chickens (Supplementary Fig. 2).

3.4. Immunogenicity of E. coli- and T. gondii-derived SAG1

The inconsistency we observed between the ELISA results for *E. coli*-derived SAG1 and western blot analysis using crude tachyzoite lysate (Figs.1 and 2) prompted us to compare the immunogenicities of *E. coli*- and *T. gondii*-derived SAG1. When comparable amounts of *E. coli*-derived and tachyzoite-derived SAG1 were western blotted with the serum sample from an experimentally infected chicken, tachyzoite-derived SAG1 reacted much more strongly with the chicken serum (Fig. 3A). The western blot analysis clearly showed that IgY reacted with SAG1 using chicken sera that had shown no reaction or a weak reaction to *E. coli*-derived SAG1 (Fig. 3B).

3.5. Antibodies in chickens with no or low parasite burdens

To facilitate parasite detection in the tissues, seven day-old chickens were infected with the recombinant *T. gondii* line expressing a red fluorescent protein (TgCatJpGi1/TaJ/GRA Red). Using a stereoscopic microscope, latent cysts from this strain in the tissues were readily detected across a wide visual field (Fig. 4A). Three out of seven infected chickens died by one week post infection with this parasite strain. Fluorescent tachyzoites were detected in the hearts, lungs, livers and brains of the dead chickens (Fig. 4B). In contrast, no cysts were detected in the hearts, brains, lungs and livers from the surviving chickens by stereoscopic microscope observation. Bioassays in mice using the organs from the surviving chickens were also negative. However, despite the inability to detect parasites, the sera from all four surviving chickens clearly reacted with tachyzoite-derived SAG1 on western blots (Fig. 4C).
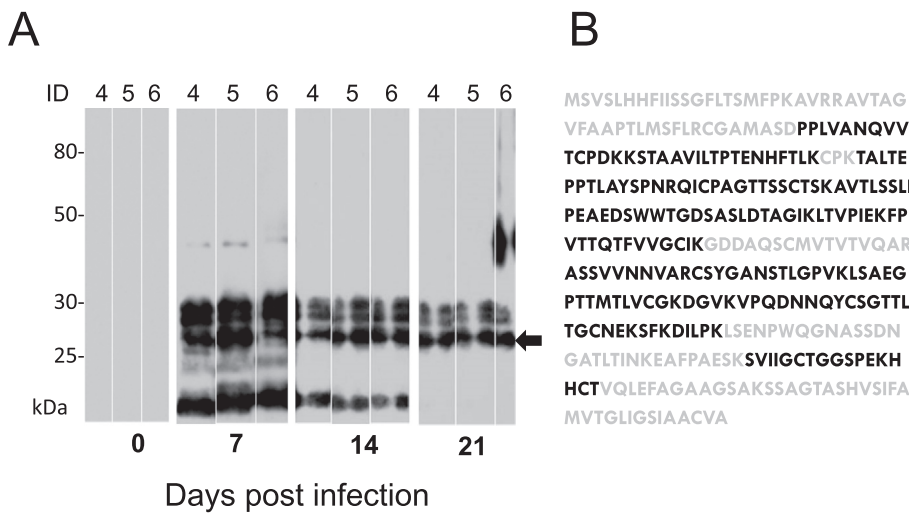
Fig. 2. Reactivity of *T. gondii*-infected chicken sera to crude *T. gondii* tachyzoite lysate. The chicken serum samples were examined by western blot analysis. A) Western blot of the chicken serum samples obtained in the experiment shown in Table 1 (chicken ID Nos. 4, 5 and 6). The antigen band that persisted throughout the entire observation period is marked by an arrow. B) Identification of the persistent band by LC-MS. Darkened letters show the identified peptide sequences that match the reported SAG1 sequence (GenBank: AAB20292.1).
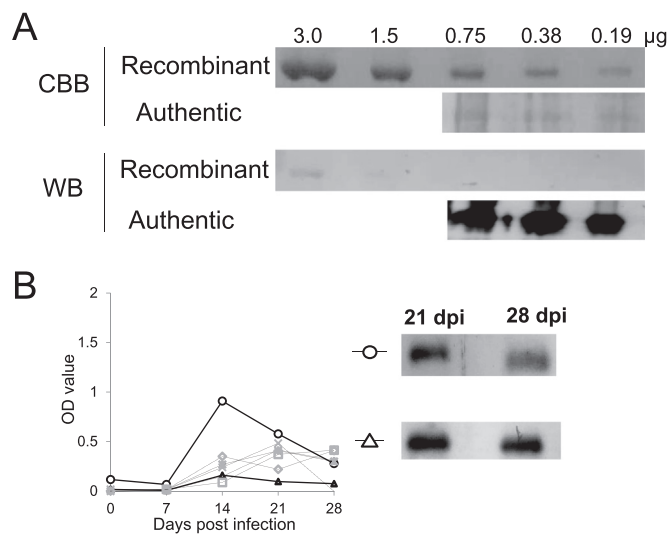
Fig. 3. Antigenicity comparison between *E. coli*-derived recombinant SAG1 and *T. gondii* tachyzoite-derived authentic SAG1. A) Coomassie brilliant blue (CBB) staining of a known concentration of recombinant SAG1 and authentic SAG1 proteins showing bands of comparable densities (upper image). Serially diluted proteins were electrophoresed on an SDS gel and stained. The electrophoresed proteins were used for western blot (WB) analysis using the sera from chickens that had been intravenously infected with 1×10^7 PLK tachyzoites. B) Reactivity of the serum samples from two infected chickens that showed no/low reactivity with the recombinant SAG1 in Fig. 1B to authentic SAG1. Western blot analysis using crude tachyzoite antigens was performed with these serum samples. dpi, day post infection.

3.6. Reactivity of field chicken sera with crude tachyzoite antigens

Serum samples collected from chickens in Ghana (Fig. 5A) were screened for reactivity to crude tachyzoite antigens. As shown in Fig. 5B, no serum samples from chickens housed in an enclosed poultry facility in LIPREC reacted with the crude tachyzoite antigens. Serum samples from the free-range chickens on a small private farm also lacked reactivity, with the exception of one chicken whose serum reacted with an antigen whose molecular weight is the putative size of SAG1 (Fig. 5B). We confirmed that the free-range chickens are regularly in contact with cats (Fig. 5A).

4. Discussion

In this study, we found that parasite-derived authentic SAG1 is the most suitable antigen for detecting *T. gondii* exposure in chickens. Once chickens are exposed to *T. gondii*, even when undetectable levels of parasites remain in their tissues, we found that their sera remained immunoreactive to the authentic SAG1 on western blot analysis using crude tachyzoite lysate as the antigen. In contrast, ELISAs with recombinant *E. coli*-produced SAG1 and a commercial latex aggregation kit (Toxocheck-MT), which is authorized by the Japanese government as a diagnostic pharmaceutical for pigs and cats, did not detect any *T. gondii* infections of more than two weeks. Although commercial latex aggregation test kits have been widely used in field surveys to obtain general information on the prevalence of *T. gondii* in chickens [15,16], these surveys might have only picked up individuals within 1–2 weeks of their infections starting and have therefore possibly underestimated the number of chickens exposed to this pathogen.

We were also interested in investigating the usefulness of western blot analysis using crude tachyzoite lysate for field research. To this end, we tested chicken serum samples collected in Ghana. All the chickens in a windowless poultry house that we examined showed no reactions with any antigen. With one exception, the chickens that we examined from the free-range farm also showed no reactions. This suggests that the indigenous micro biota in field chickens do not cause non-specific and/or cross reactions with crude tachyzoite antigens. The chicken that tested positive based on its western blot reaction could not be analyzed further; however, based on the non-specific reactions of the other chicken sera, and the size of the observed band, which corresponds to the putative size of SAG1, and coupled with the farm environment where cats have free access, it seems likely that the chicken on this farm is infected with *T. gondii* parasites. We also ascertained that for chickens experimentally infected with tachyzoites, no parasites were observable in any of the organs examined, and that bioassay of the organs also proved negative. These results suggest that experimental tachyzoite infection in chickens result in the formation of a very small number of cysts or all the parasites are cleared by the chicken's immune response and so no cysts are formed. The injected tachyzoites induced an immune-response and the production of antibodies during the acute phase of infection, and the elevated antibody titers were maintained even after the acute phase thus western blot analysis showed that all sera were positive for *T. gondii* antibodies. Western blot analysis could be a very useful tool for field surveillance of chickens exposed to *T. gondii*. This idea is further supported by the fact that western blot analysis was able to determine seroprevalence in tachyzoite-infected chickens, even when no parasites were observable. Such an approach would be especially useful in situations where high sensitivity is required; for example, to detect environmental levels of *Toxoplasma*.

We also found that parasite-derived authentic SAG1 is much more
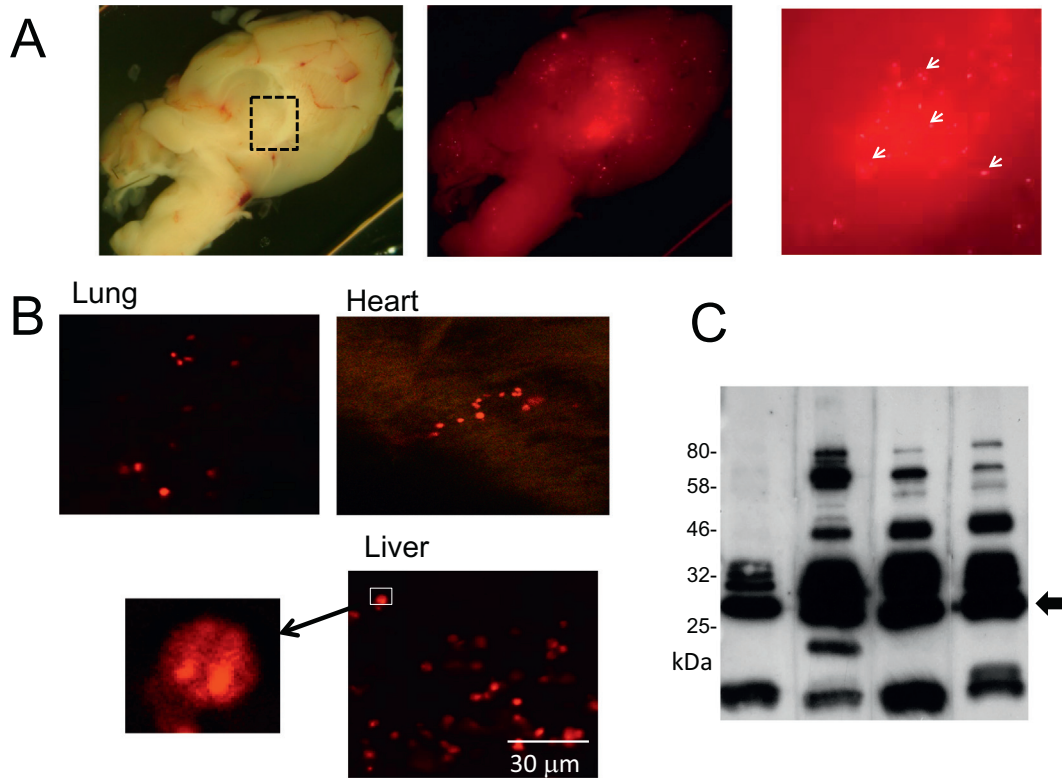
Fig. 4. Parasite burden and antibody production in infected chickens. A) Low magnification image of a mouse brain harboring cysts from a recombinant parasite expressing the red fluorescent protein TgCatJpGi1/TaJ GRA Red. B) TgCatJpGi1/TaJ GRA Red tachyzoites in the lung, heart and liver from a dead chicken. C) Serum sample reactivity for four experimentally infected chickens with low or no parasite burdens, to crude tachyzoite antigens. Western blot analysis using a crude tachyzoite lysate was performed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
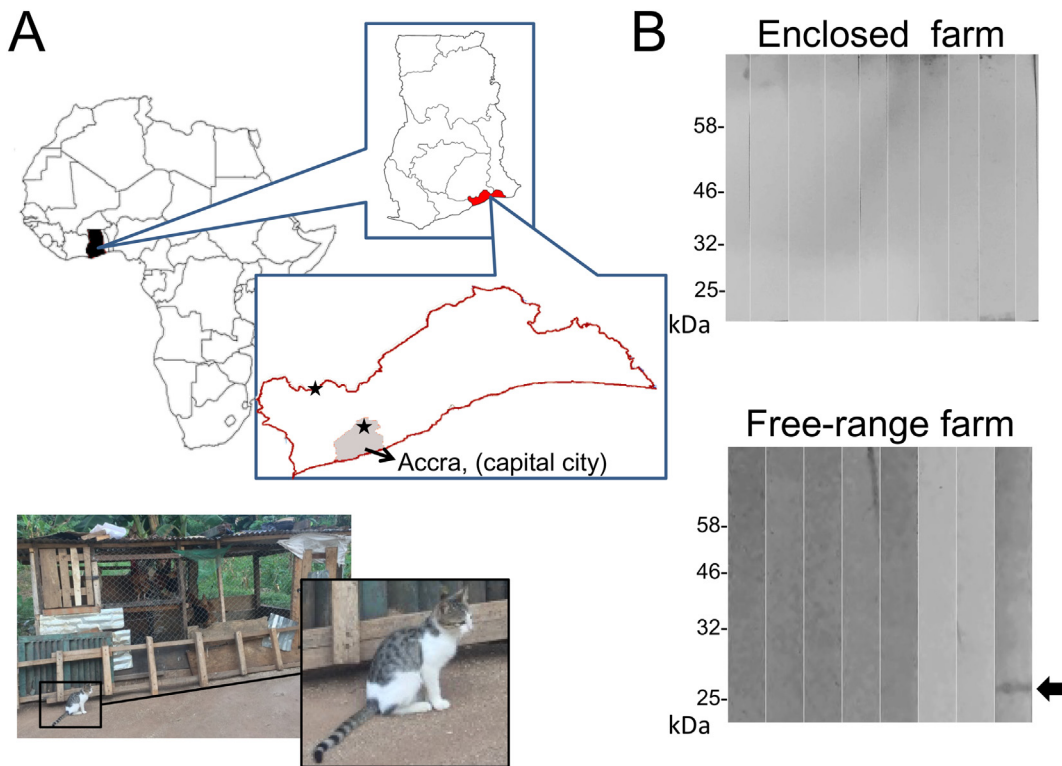
Fig. 5. Reactivity testing of the serum samples collected from two different types of chicken farm in Ghana, Africa, to crude tachyzoite lysate. A) Sample collection sites (upper) and the farm in Nsawam where the free range chicken samples were collected. B) Reactivity of the field-collected serum samples to crude tachyzoite lysate on western blots. Arrow indicates the reactive band.

immunogenic in chickens than recombinant SAG1 produced in *E. coli*. A similar phenomenon was also seen in mice [5]. Several field-based surveillance studies on *T. gondii* in chickens using the Modified Agglutination Test (MAT), a method used to detect antibodies that react with surface antigens of fixed, whole tachyzoites, have reported on the high prevalence of this parasite in many parts of the world [1]. Considering that SAG1 is a major surface antigen in *T. gondii* tachyzoites, the high immunogenicity of parasite-derived authentic SAG1 might contribute to the high sensitivity of MAT. MAT has been widely used in academic studies to identify *T. gondii*-infected chickens [17,18], and has also been validated with experimentally-infected chickens [19]. However, MAT is limited in its scope for large-scale field surveillance because large numbers of fixed tachyzoites are required. To overcome this problem, it would be helpful to understand why *E. coli*-produced recombinant SAG1 has low immunogenicity and establish a methodology to produce recombinant SAG1 that better reflects the high immunogenicity seen with the native protein. Currently, to effectively produce recombinant SAG1 in *E. coli*, it is usually expressed in a truncated form with the GPI anchor missing. Hence, it is possible that the GPI-anchored domain is required for the diagnostic integrity of SAG1 when applied to chicken infections.

In conclusion, the data presented herein support the use of authentic SAG1 as a diagnostic antigen for *T. gondii* surveillance in chickens. Although parasite-derived SAG1 is necessary for surveillance at present, if an effective and inexpensive method to produce large amounts of highly immunogenic recombinant SAG1 protein is established in the near future, this would be a great contribution to the field surveillance of *T. gondii* in chickens. In addition, to be able to definitively determine the relationship between parasite burden and seroprevalence, the use of tissue cysts or oocysts will be appropriate in further experiments, and the current diagnostic system should be compared with the MAT.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parint.2019.01.001.

References

[1] J.P. Dubey, Toxoplasma gondii infections in chickens (Gallus domesticus): prevalence, clinical disease, diagnosis and public health significance, Zoonoses Public Health 57 (2010) 60–73, https://doi.org/10.1111/j.1863-2378.2009.01274.x.

[2] J.L. Jones, D. Kruszon-Moran, M. Wilson, G. McQuillan, T. Navin, J.B. McAuley, Toxoplasma gondii infection in the United States: seroprevalence and risk factors, Am. J. Epidemiol. 154 (2001) 357–365, https://doi.org/10.1093/aje/154.4.357.

[3] J.P. Dubey, T. Lehmann, F. Lautner, O.C.H. Kwok, H.R. Gamble, Toxoplasmosis in sentinel chickens (Gallus domesticus) in New England farms: Seroconversion, distribution of tissue cysts in brain, heart, and skeletal muscle by bioassay in mice and cats, Vet. Parasitol. 214 (2015) 55–58, https://doi.org/10.1016/j.vetpar.2015.09.004.

[4] G. Moré, P. Maksimov, L. Pardini, D.C. Herrmann, D. Bacigalupe, A. Maksimov, W. Basso, F.J. Conraths, G. Schares, M.C. Venturini, Toxoplasma gondii infection in sentinel and free-range chickens from Argentina, Vet. Parasitol. 184 (2012) 116–121, https://doi.org/10.1016/j.vetpar.2011.09.012.

[5] D. Harning, J. Spenter, A. Metsis, J. Vuust, E. Petersen, Recombinant Toxoplasma gondii surface antigen 1 (P30) expressed in Escherichia coli is recognized by human Toxoplasma-specific immunoglobulin M (IgM) and IgG antibodies, Clin. Diagn. Lab. Immunol. 3 (1996) 355–357.

[6] I. Inoue, C.S. Leow, D. Husin, K. Matsuo, P. Darmani, A survey of Toxoplasma gondii antibodies in pigs in Indonesia, Southeast Asian J. Trop. Med. Public Health 32 (2001) 38–40.

[7] A.E. Abdelbaset, H. Alhasan, D. Salman, M.H. Karram, M.A. Ellah Rushdi, X. Xuenan, M. Igarashi, Evaluation of recombinant antigens in combination and single formula for diagnosis of feline toxoplasmosis, Exp. Parasitol. 172 (2017) 1–4, https://doi.org/10.1016/j.exppara.2016.11.003.

[8] A. Hotop, S. Buschtöns, B. Bangoura, B. Zöller, M. Koethe, K. Spekker-Bosker, S.K. Hotop, A.M. Tenter, W. Däubener, R.K. Straubinger, U. Groß, Humoral immune responses in chickens and turkeys after infection with Toxoplasma gondii by using recombinant antigens, Parasitol. Res. 113 (2014) 1473–1480, https://doi.org/10.1007/s00436-014-3788-x.

[9] R.G. Donald, D.S. Roos, Stable molecular transformation of Toxoplasma gondii: a selectable dihydrofolate reductase-thymidylate synthase marker based on drug-resistance mutations in malaria, Proc. Natl. Acad. Sci. 90 (1993) 11703–11707, https://doi.org/10.1073/pnas.90.24.11703.

[10] Y. Nishikawa, X. Xuenan, L. Makala, O. Vielemeyer, K.A. Joiner, H. Nagasawa, Characterisation of Toxoplasma gondii engineered to express mouse interferon-gamma, Int. J. Parasitol. (2003), https://doi.org/10.1016/S0020-7519(03)00204-2.

[11] Y. Taniguchi, C. Appiah-Kwarteng, M. Murakami, J. Fukumoto, K. Nagamune, T. Matsuo, T. Masatani, H. Kanuka, T. Hoshina, K. Kitoh, Y. Takashima, Atypical virulence in a type III Toxoplasma gondii strain isolated in Japan, Parasitol. Int. 67 (2018) 587–592, https://doi.org/10.1016/j.parint.2018.05.010.

[12] E.N. Kimbita, X. Xuan, X. Huang, T. Miyazawa, S. Fukumoto, M. Mishima, H. Suzuki, C. Sugimoto, H. Nagasawa, K. Fujisaki, N. Suzuki, T. Mikami, I. Igarashi, Serodiagnosis of Toxoplasma gondii infection in cats by enzyme-linked immunosorbent assay using recombinant SAG1, Vet. Parasitol. 102 (2001) 35–44, https://doi.org/10.1016/S0304-4017(01)00522-2.

[13] M.A. Terkawi, K. Kameyama, N.H. Rasul, X. Xuan, Y. Nishikawa, Development of an immunochromatographic assay based on dense granule protein 7 for serological detection of toxoplasma gondii infection, Clin. Vaccine Immunol. 20 (2013) 596–601, https://doi.org/10.1128/CVI.00747-12.

[14] J.P. Dubey, Refinement of pepsin digestion method for isolation of Toxoplasma gondii from infected tissues, Vet. Parasitol. 74 (1998) 75–77, https://doi.org/10.1016/S0304-4017(97)00135-0.

[15] K. Matsuo, R. Kamai, H. Uetsu, H. Goto, Y. Takashima, K. Nagamune, Seroprevalence of Toxoplasma gondii infection in cattle, horses, pigs and chickens in Japan, Parasitol. Int. 63 (2014) 638–639, https://doi.org/10.1016/j.parint.2014.04.003.

[16] N. Zia-Ali, A. Fazaeli, M. Khoramizadeh, D. Ajzenberg, M. Dardé, H. Keshavarz-Valian, Isolation and molecular characterization of Toxoplasma gondii strains from different hosts in Iran, Parasitol. Res. 101 (2007) 111–115, https://doi.org/10.1007/s00436-007-0461-7.

[17] C.M. Hamilton, P.J. Kelly, K. Boey, T.M. Corey, H. Huynh, D. Metzler, I. Villena, C. Su, E.A. Innes, F. Katzer, Predominance of Atypical Genotypes of Toxoplasma Gondii in Free-Roaming Chickens in St. Kitts, West Indies, Parasites and Vectors, vol. 10, (2017), pp. 1–9, https://doi.org/10.1186/s13071-017-2019-6.

[18] G. Tilahun, N. Tiao, L.R. Ferreira, S. Choudhary, S. Oliveira, S.K. Verma, O.C.H. Kwok, B. Molla, W.J.A. Saville, G. Medhin, T. Kassa, H. Aleme, W.A. Gebreyes, C. Su, J.P. Dubey, Prevalence of *Toxoplasma gondii* from free-range chickens (*Gallus domesticus*) from Addis Ababa, Ethiopia, J. Parasitol. 99 (2013) 740–741, https://doi.org/10.1645/12-25.1.

[19] C. Yan, C.L. Yue, Z.G. Yuan, R.Q. Lin, Y. He, C.C. Yin, M.J. Xu, H.Q. Song, X.Q. Zhu, Molecular and serological diagnosis of Toxoplasma gondii infection in experimentally infected chickens, Vet. Parasitol. 173 (2010) 179–183, https://doi.org/10.1016/j.vetpar.2010.07.011.