The Journal of Veterinary Medical Science

FULL PAPER

Parasitology

Diagnostic value of recombinant nanoluciferase fused *Toxoplasma gondii* antigens in Luciferase-linked Antibody Capture Assay (LACA) for *Toxoplasma* infection in pigs

Hieu Duc DUONG[1,2], Yuji TANIGUCHI[3], Yasuhiro TAKASHIMA[3], Satoshi SEKIGUCHI[4,5], Khin Myo AYE[6,7], Parnian AHMADI[1], Linh Khanh BUI[2], Takao IRIE[1,5], Eiji NAGAYASU[6] and Ayako YOSHIDA[1,5]*

[1)]Laboratory of Veterinary Parasitic Diseases, Department of Veterinary Sciences, Faculty of Agriculture, University of Miyazaki, Miyazaki, Japan
[2)]Department of Veterinary Parasitology, Faculty of Veterinary Medicine, Vietnam National University of Agriculture, Hanoi, Vietnam
[3)]Department of Veterinary Parasitology, Faculty of Applied Biological Sciences, Gifu University, Gifu, Japan
[4)]Laboratory of Animal Infectious Disease and Prevention, Department of Veterinary Sciences, Faculty of Agriculture, University of Miyazaki, Miyazaki, Japan
[5)]Centre for Animal Diseases Control (CADIC), University of Miyazaki, Miyazaki, Japan
[6)]Division of Parasitology, Department of Infectious Diseases, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan
[7)]Parasitology Research Division, Department of Medical Research, Yangon, Myanmar

ABSTRACT. Toxoplasmosis is a widespread protozoan zoonosis. Since ingesting undercooked meat harboring *Toxoplasma gondii* cyst is considered one of the major transmission routes to humans, the screening of *T. gondii* in meat-producing animals can reduce the risk of food-borne toxoplasmosis in humans. Among serological diagnostic methods, Luciferase-linked Antibody Capture Assay (LACA) has been found to be a promising platform with high sensitivity and specificity. In this study, we aimed to evaluate recombinant nanoluciferase fused-*T. gondii* antigens (rNluc-GRA6, rNluc-GRA7, rNluc-GRA8 and rNluc-BAG1) for their potential use in LACA for pigs. As a result, the sensitivity of GRA6-, GRA7-, GRA8- and BAG1-LACA were 70.0%, 80.0%, 80.0% and 30.0% with specificity 87.0%, 81.5%, 74.1% and 50.0%, respectively. The cocktail LACA using a mixture of rNluc-GRA6, rNluc-GRA7 and rNluc-GRA8 indicated higher sensitivity (90.0%) and a similar specificity (96.3%) in comparison with the commercial ELISA kit. Compared to the Dye-Test as a reference test, cocktail LACA showed strong agreement (kappa value=0.811) when we assessed pig sera collected at the slaughterhouse. In addition, we also successfully established the rapid LACA format for the detection of *Toxoplasma* infection in pigs (called Rapid-LACA) in which the test could be performed within 30 min. In Rapid-LACA, the protein A pre-coated/blocked plates could be preserved at −30°C, 4°C or room temperature conditions for at least two months without compromising on the quality of assay.

KEYWORDS: Luciferase-linked Antibody Capture Assay (LACA), nanoluciferase, pig, serodiagnosis, *Toxoplasma gondii*

J. Vet. Med. Sci.
84(7): 905–913, 2022
doi: 10.1292/jvms.22-0099

Received: 7 March 2022
Accepted: 6 May 2022
Advanced Epub:
18 May 2022

An estimated 30% of the global pig population has been exposed to toxoplasmosis, one of the most common parasitic zoonosis, caused by the obligate intracellular protozoan *Toxoplasma gondii* [15]. Through the consumption of raw or undercooked pork contaminated with *T. gondii* tissue cysts, humans may get infected [21]. Immunodeficient patients, as well as pregnant women, have been considered as a category at higher clinical risk. Particularly, *T. gondii* infection may cause encephalitis, pneumonia, and disseminated infection and generate severe problems with spontaneous abortion, fetal demise or neonatal malformations,

*Correspondence to: Yoshida, A.: kukuri@med.miyazaki-u.ac.jp, Laboratory of Veterinary Parasitic Diseases, Department of Veterinary Sciences, Faculty of Agriculture, University of Miyazaki, 1-1 Gakuen-Kibanadai Nishi, Miyazaki 889-2192, Japan

neurological damage, blindness in newborns [39].

Since pork containing *T. gondii* cysts can be a potential source of human infection, the diagnosis of toxoplasmosis in pigs will continue to be very important for limiting the risk of transmission to humans. The detection of *T. gondii* through direct visualization is impossible by currently practiced meat inspection. However serological tests for specific antibody detection can be useful to determine potentially contaminated pork as well as to monitor biosecurity measures in pig farms [36]. Therefore, reliable serodiagnostic tests for *T. gondii* infection in pigs are crucially required.

There are numerous serological tests routinely used for diagnosis of *T. gondii* infection in pigs such as Dye-Test, modified agglutination test (MAT), indirect hemagglutination (IHA), latex agglutination test (LAT), indirect fluorescent antibody test (IFAT) and ELISA [11]. Some of them are commercially available, however, these kits used whole tachyzoite lysate as antigens that require time and are labor-consuming to produce. Consequently, commercial kits are generally costly and available for research purposes only. Moreover, using tachyzoite antigen may lead to inter-assay variation and difficulty in standardization of the tests [3, 5].

To overcome the disadvantages of using tachyzoite lysate antigens, several recombinant antigens were evaluated for their potential use mostly in conventional ELISA for pigs. In these previous studies, the assessment of the sensitivity and specificity of ELISA tests limited in comparison with MAT or LAT as reference tests [4, 15, 22, 31, 40], despite the fact that the sensitivity and specificity were relatively low; 82.9% and 90.29% for MAT, 45.9% and 96.9% for LAT when compared to the isolation of the viable *T. gondii* from the tissue of pigs [12, 35].

Previously, a novel Luciferase-linked Antibody Capture Assay (LACA) utilizing recombinant nanoluciferase fused *T. gondii* antigen was developed for the diagnosis of *T. gondii* infection in chickens with unexpectedly high sensitivity and specificity [13]. As reported, LACA has been considered a promising diagnostic platform owing to its several advantages as compared to other serological assays such as enzyme-linked immunosorbent assay (ELISA). In LACA, the nanoluciferase-fusion antigens are not only used as bait to capture the target-specific antibodies, but also as ultrasensitive probes. Accordingly, luminescent signals are directly reported from luciferase-fusion antigens as detectors without using the conjugated secondary antibodies that may lead to cross-reaction and high background due to non-specific binding which is a common problem in conventional ELISA. The other advantage of LACA over ELISA includes the fact that the lysates of plasmid-transfected cells can be directly applied without purification procedures, thus, significantly reducing the time and effort required to produce the target antigens [13, 24].

As with other serological assays, both the sensitivity and specificity of LACA depend on the choice of candidate antigens. From a diagnostic point of view, the *T. gondii* dense granule proteins (GRAs), which are exocytosed into the parasitophorous vacuole (PV) and integrated into the PV membrane (PVM) where they interact with host cell components, were generally thought of as potential diagnostic markers [27]. Among them, GRA6, GRA7, and GRA8 have been widely employed in ELISA for the detection of anti-*T. gondii* antibodies in animals, including pigs [14]. In addition, the bradyzoite specific cytoplasmic antigen (BAG1) has been found to induce humoral and cell-mediated immune responses early after infection [10].

Therefore, in this study we have evaluated the usefulness of recombinant nanoluciferase fused *T. gondii* antigens such as rNluc-GRA6, rNluc-GRA7, rNluc-GRA8 and rNluc-BAG1 in the LACA platform. In addition, we aimed to establish a modified version of LACA for rapid and simple practical application which was termed Rapid-LACA.

MATERIALS AND METHODS

Pig sera

Ten 5-month-old female micro-mini pigs purchased from Fuji Micra Inc. (Fujinomiya, Japan) were orally infected with *T. gondii* cysts or intraperitoneally injected with tachyzoites of strains TgCatJpGi1/TaJ or TgCatJpOk4 [37, 38]. Briefly, TgCatJpGi1/TaJ cysts were maintained by serial passage in C57BL/6 mice by brain cyst inoculation. After 14 days post infection, the brains of the surviving mice were harvested and homogenized. The number of cysts in the brain emulsion was counted. Four micro mini-pigs were given 900 TgCatJpGi1/TaJ cysts mixed in with their food. On the other hand, TgCatJpOk4 tachyzoites were maintained in the Vero cell cultures and collected through a 27 G needle then purified through a five-micrometer filter. The number of parasites was counted using a cell counter, and 1×10^7 tachyzoites were injected intraperitoneally into each six micro-mini pigs. After infection, symptoms and body temperature were observed and recorded periodically. The serum samples were collected within the time frame of 10 to 140 days post infection (DPI) and stored at −30°C until the time of analysis. All sera were seropositive for tachyzoite antigens in Western blotting [37]. Additionally, the sera from Darnish crossbred pigs experimentally innoculated with 10,000 *Toxocara cati* (n=7) or *Ascaris suum* (n=9) embryonated eggs using a stomach tube as well as the negative sera from uninfected healthy pigs (n=38) were used to evaluate the specificity of the assay. Pigs were purchased from a specific pathogen free farm in Denmark, and were tested negative for helminth eggs and oocysts in the feces before experimental infection. Blood samples were collected 4 weeks after infection and sera were stored at −30°C until use. Animal experiments were approved by the ethics committee of Gifu University (15078) and the Animal Experiments Inspectorate, Ministry of Justice, Denmark (2000/561-321) and conducted in accordance with applicable guidelines.

Ninety-nine pig sera were collected from growing-finishing pigs aged over 6 months at two slaughterhouses located on Kyushu Island, Japan from June to July 2014, which were kept in a laboratory, were also applied in this study [22].

Parasites and Dye-Test

T. gondii RH strain tachyzoites were maintained *in vitro* in confluent primary human foreskin fibroblasts (HFFs) grown in

Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% GibcoBRL fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA, USA) and Gibco™ Antibiotic-Antimycotic (100X) (Thermo Fisher, Waltham, MA, USA) at 37°C with 5% CO_2.

The tachyzoites were harvested, purified using 2 µm Whatman™ filter (GE Healthcare, Chicago, IL, USA) then used for the Sabin-Feldman Dye-Test, which has been refered to as a "gold standard" test for the detection of anti-*T. gondii* antibodies in humans [29, 31] as well as a reliable serodiagnostic measure for toxoplasmosis in swine [17]. The Dye-Test was performed in accordance with the protocol described previously [19]. The cut-off >4 IU was defined as a positive for the Dye-Test in pigs [4].

Indirect enzyme-linked immunosorbent assay (Commercial ELISA)

The Porcine Toxoplasma ELISA kit (Nittobo Medical, Tokyo, Japan) was performed according to the manufacturer's instructions. Each serum sample was independently tested in duplicate at a dilution of 1:100. The optical density (OD) was measured at 450 nm in xMark Spectrophotometer (Bio-Rad Laboratories, Inc., Hecules, CA, USA) and the results were interpreted by calculating the ratio of the sample OD to the average of the positive control (S/P), (S/P=Sample OD–Negative control OD/ Positive control OD–Negative control OD). A S/P ratio greater than 0.15 was regarded as positive as suggested by the manufacturer.

Preparation of recombinant nanoluciferase-fused T. gondii antigens

Four recombinant nanoluciferase fused *T. gondii* (RH strain) antigens (rNluc-GRA6, rNluc-GRA7, rNluc-GRA8 and rNluc-BAG1) were used in this study. Each antigen encoding sequence was constructed and sub-cloned into the pET100/D-TOPO vector (Invitrogen, Carlsbad, CA, USA), then expressed in *Escherichia coli* BL21 star cells (DE3) (Invitrogen). The recombinant antigens, expressed as fusion proteins with a 6xHis tag, were purified using Ni-NTA (Wako Life Science, Osaka, Japan) as previously described [2, 13]. The concentrations of the recovered proteins were determined using Qubit 4 Fluorometer (Invitrogen), and aliquots were stored at −80°C.

Luciferase-linked antibody capture assay (LACA)

The LACA assays were performed as described by Duong *et al.* (2020) with some modifications [13]. In brief, the protein A (2 µg/ml) (Funakoshi Co., Ltd., Tokyo, Japan) was immobilized onto the white OptiPlate-96 HB microplates (PerkinElmer, Waltham, MA, USA) overnight at 4°C in coating buffer (0.05 M carbonate/bicarbonate buffer, pH 9.6). In order to block non-specific protein binding, 200 µl of 1% Casein in phosphate-buffered saline (PBS)-Tween 20 (0.05%) was added to each well, and the plate was then incubated for 2 hr at 37°C. After washing the plates three times with PBS-Tween 20 (0.05%) using Nunc-Immuno™ microplate washer (Nunc, Roskilde, Denmark), the diluted serum samples (1:10) were loaded and incubated at 37°C for 1 hr. Next, the plates were washed three times and incubated with 0.5 µg/ml of each recombinant antigens (rNluc-GRA6, rNluc-GRA7, rNluc-GRA8, rNluc-BAG1) for 1 hr at 37°C. In addition, rNluc-GRA6, rNluc-GRA7 and rNluc-GRA8 were mixed together in equal proportion to prepare a cocktail antigen, which was also applied at this step with a total concentration of 1.5 µg/ml. Thereafter, the plates were washed four times before the diluated (1:1,000) Nano-Glo subtrate in 0.1% Bovine serum albumin (BSA)-PBS was added to each well. Finally, the luciferase activity was measured using VICTOR^3 Multilabel Plate Reader (PerkinElmer) and the data were presented as the ratio of luciferase activity of each sample to the luciferase activity of negative serum (S/N ratio). Each serum sample was independently assayed in duplicate and the positive as well as negative controls from pooled sera of experimentally infected and uninfected pigs were included on each plate to normalize the data of different LACA batches.

Rapid-LACA

The wells of 18 of the white OptiPlate-96 HB microplates (PerkinElmer) were coated with protein A (2 µg/ml) in coated buffer solution at 4°C overnight. After washing, the plates were blocked with 200 µl of Blocking Reagent for ELISA- Chemically Defined- (Cosmo Bio, Tokyo, Japan) and incubated at room temperature (RT) for 2 hr. Thereafter, the plates were completely drained, air dried at 37°C for 4 hr and sealed. Lastly, all of the plates were subdivided into three groups comprising of six plates each, and stored at RT, 4°C and −30°C.

Each diluted serum sample (1:10) and the above described cocktail antigen were mixed together in a final volume of 50 µl. Next, the mixture was dispensed into each well of the protein A pre-coated/blocked plates, incubated for 30 min at 37°C and then washed four times with PBS-Tween 20 (0.05%). Lastly, Nano-Glo subtrate was added to each well followed by measurement of luciferase activity as mentioned above. Schematic diagram for procedure of LACA and Rapid-LACA was described in Fig. 1. Ninety pig serum samples were applied for Rapid-LACA and standard LACA, then the results obtained from both tests were compared with each other.

Validation of the stability of the protein A pre-coated/blocked plates stored at different conditions

The protein A pre-coated/blocked plates were subdivided into three groups of six plates, and kept at RT, 4°C and −30°C. The stability of the plates used for Rapid-LACA was examined at different storage times (on day 0, day 10, day 20, day 30, day 40, and day 60) with positive (n=5) and negative (n=5) serum samples. Each serum sample was independently performed in triplicate. The resulting data of Rapid-LACA was compared and expressed as the ratio of luciferase activity of positive sample to the luciferase activity of negative serum (P/N ratio).

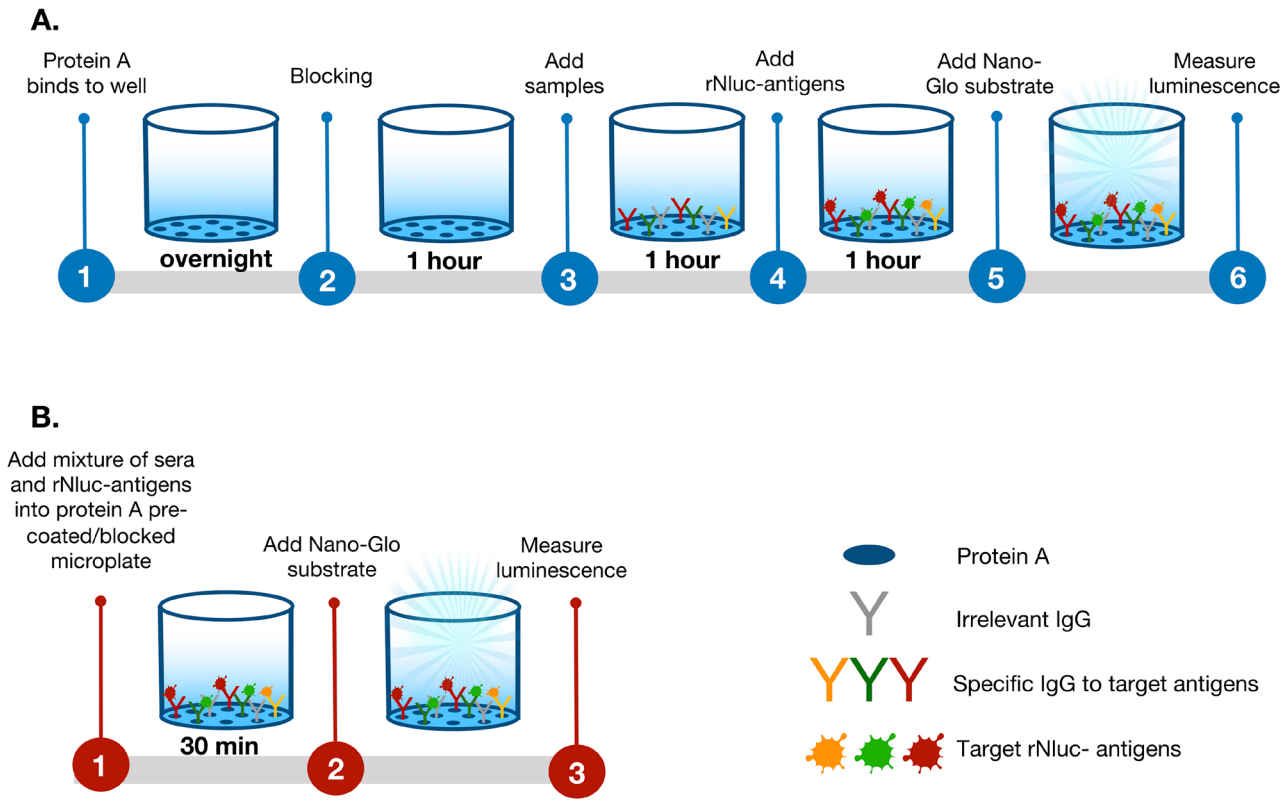

Fig. 1. Schematic representation of Luciferase-linked Antibody Capture Assay (LACA) and Rapid-LACA. For a LACA (**A**), a microplate is coated overnight with protein A. After 1 hr blocking, serum samples are added and incubated for 1 hr at 37°C, follow then rNluc- antigens are loaded to the microplate wells and incubated at 37°C. After washing, Nano-Glo substrates are added to detect the luminescent signals. For a Rapid-LACA (**B**), a protein A pre-coated/blocked plate is used. The rNluc-antigens and sera were co-incubated for 30 min at 37°C. After washing and adding Nano-Glo substrate, the luminescent signals are measured by a luminometer.

Statistical analysis

All statistical calculations were carried out using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Receiver operator characteristic (ROC) analysis was used to optimize the cut-off values of LACA that maximize both diagnostic sensitivity and specificity. The agreement between LACA, ELISA, and Dye-Test, which were applied for a set of pig sera collected from the field, was estimated by kappa value with 95% confidence interval (95% CI). According to the interpretation of Cohen's kappa, a value of ≥0.75 represents excellent agreement, 0.40 to 0.75 represents fair to good agreement, and <0.40 represents poor agreement [16]. In addition, the value of Rapid-LACA at each storage time point was defined by the mean value of ratio between luciferase activity of positive sera and the luciferase activity of negative sera (P/N ratio) ± 1 standard deviation. The statistical differences between the groups were analyzed by Mann-Whitney test and considered significant at P-value <0.05. In order to ensure that the results obtained from different pre-coated/blocked plates preserved at different conditions in Rapid-LACA were consistent after a storage period of 60 days, Pearson's rank correlation coefficient was calculated to determine the correlations between the plates kept at RT, 4°C and −30°C and standard LACA. The value of Pearson's rank correlation coefficient was interpreted regarding the rule of Thumb [26].

RESULTS

Assessment of diagnostic performance of LACA in experimentally T. gondii infected pigs

The distribution of S/N ratios by LACA utilizing different recombinant nanoluciferase was shown in Fig. 2. The optimal cut-off values by the ROC curves analysis demonstrated that S/N ratio of 1.161 for rNluc-GRA6, 1.142 for rNluc-GRA7, 1.128 for rNluc-GRA8 and 1.030 for rNluc-BAG1 were capable of distinguishing between the positive and negative signals with discrimination capacity AUC of 0.83, 0.87, 0.78 and 0.57, respectively (Fig. 3, Table 1). Regarding our results, rNluc-BAG1 exhibited relatively weak antibody responses whereas rNluc-GRA6, rNluc-GRA7 and rNluc-GRA8 gave a better diagnostic perfomance. However, the sensitivity and specificity of GRA6-LACA, GRA7-LACA and GRA8-LACA were limited at a range between 70.0–80.0% and 74.1–84.4% with the accuracy between 75.0–84.4%. At the recommended cut-off by the manufacturer, the commerical Porcine Toxoplasma ELISA kit possessed the highest specificity of 98.2% (95% CI: 94.6–100.0%) in contrast to the lowest sensitivity, when there were only three out of ten experimentally *T. gondii* infected pigs that could be detected as positives. Expectedly, the

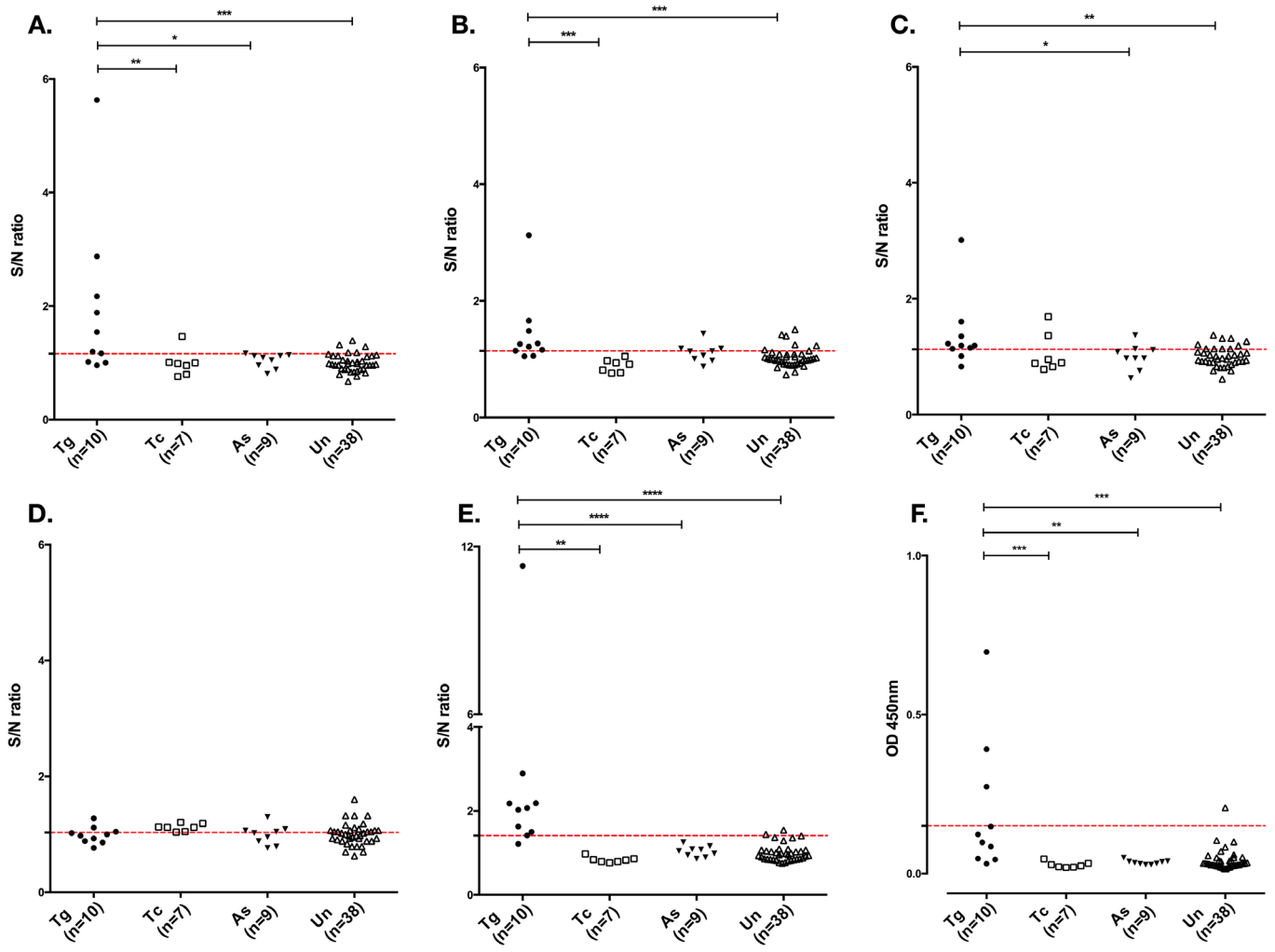

Fig. 2. Diagnostic performance of Luciferase-linked Antibody Capture Assay (LACA) using rNluc-fused *Toxoplasma gondii* antigens and commercial enzyme-linked immunosorbent assay (ELISA). LACA ultilizing rNluc-GRA6 (**A**), rNluc-GRA7 (**B**), rNluc-GRA8 (**C**), rNluc-BAG1 (**D**) or antigen mixture of rNluc-GRA6, 7, 8 (**E**) and ELISA (**F**) were perfomed by *Toxoplasma gondii* (Tg), *Toxocara cati* (Tc) and *Ascaris suum* (As) infected and uninfected (Un) pig sera. The y-axis displayed the ratio of luciferase activity of each sample to the luciferase activity of negative serum (S/N ratio) obtained from LACA or the absorbance (OD) value from ELISA. Broken lines denoted the cut-off value calculated by receiver operating characteristic (ROC) analysis. Regarding Mann-Whiney's test, the significant differences between each group were marked by asterisks (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001).

ROC curve of the LACA using the cocktail antigen that included rNluc-GRA6, rNluc-GRA7 and rNluc-GRA8 together (Fig. 3), further indicated the relatively high sensitivity (90.0%, 95% CI: 71.4–100.0%) and specificity (96.3%, 95% CI: 91.2–100.0%) with the accuracy of 95.3% (Table 1).

Correlation of LACA with Dye-Test in naturally T. gondii exposed pigs

Ninety-nine pig sera collected from slaughterhouses were examined for *T. gondii* using Dye-Test as a reference. Among them, 21 positive samples and 78 negative samples were determined. The same set of these sera was applied for both LACA and commercial ELISA. The agreement between the "gold standard" Dye-Test and other assays was evaluated by inter-rater agreement (Kappa) test (Table 2). The kappa values ranged between 0.024–0.415 for LACA using separately single antigens as well as commercial ELISA, confirming the fairlypoor agreement with Dye-Test. Meanwhile, LACA using the cocktail antigen (Cocktail LACA) showed a strong agreement with a kappa value of 0.811 (95% CI: 0.679–0.944).

Assessment of Rapid-LACA and stability of pre-coated/blocked plates

Since the combination of rNluc-GRA6, 7, 8 antigens was shown as a potential diagnostic marker in standard LACA for *T. gondii* infection in pigs, we used this cocktail antigen for further evaluatation of its applicability in Rapid LACA (Fig. 4). The values of each individual serum samples obtained from Rapid LACA showed significantly positive correlations to standard LACA with the Pearson correlation coefficient of 0.887 (95%CI: 0.834–0.925, P<0.001). In order to investigate the effects of protein A pre-coated/

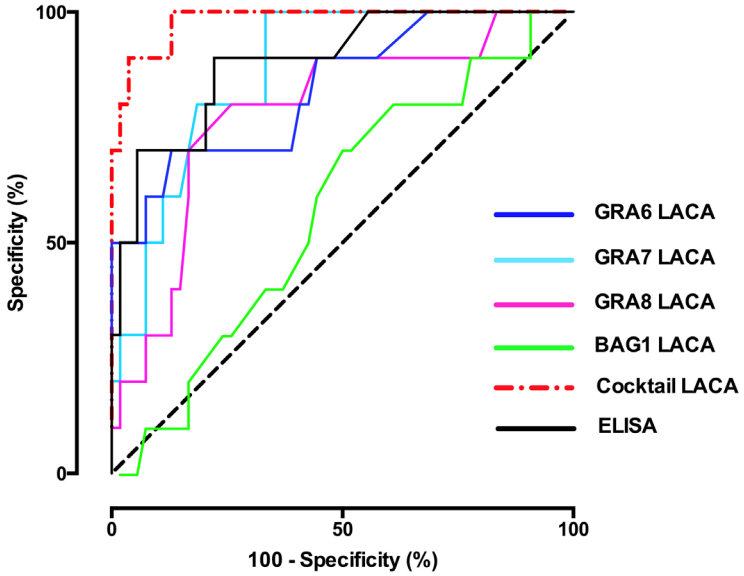

Fig. 3. Receiver operating characteristic (ROC) curves obtained from the analysis Luciferase-linked Antibody Capture Assay (LACA) and enzyme-linked immunosorbent assay (ELISA) data from experimental pig serum samples. ROC curves were used to determine the cut-off values and area under the curve (AUC) for LACA and ELISA. The non-discrimination border with AUC of 0.5 is represented by a dotted line. The sensitivity (%) is displayed on the y-axis and 100-specificity (%) on the x-axis.

Table 1. Evaluation of different rNluc-fused *Toxoplasma gondii* antigens in Luciferase-linked Antibody Capture Assay (LACA) in comparison with enzyme-linked immunosorbent assay (ELISA)

	No. tested	No. positive (%)					
		LACA					Commercial ELISA
		GRA6	GRA7	GRA8	BAG1	Cocktail	
Toxoplasma gondii	10	7 (70.0)	8 (80.0)	8 (80.0)	3 (30.0)	9 (90.0)	3 (30.0)
Toxocara cati	7	1 (14.3)	0 (0.0)	2 (28.6)	7 (100.0)	0 (0.0)	0 (0.0)
Ascaris suum	9	1 (14.3)	3 (33.3)	2 (22.2)	4 (44.4)	0 (0.0)	0 (0.0)
Uninfected	38	5 (13.2)	7 (18.4)	10 (26.3)	16 (42.1)	2 (5.3)	1 (2.6)
Cut-off by ROC (S/N ratio)		1.161	1.142	1.128	1.03	1.412	0.150 *
AUC ± SEM		0.83 ± 0.07	0.87 ± 0.05	0.78 ± 0.08	0.57 ± 0.09	0.98 ± 0.02	0.89 ± 0.06
P value		<0.01	<0.01	<0.01	0.47	<0.01	<0.01
Sensitivity (%)		70.0	80.0	80.0	30.0	90.0	30.0
Specificity (%)		87.0	81.5	74.1	50.0	96.3	98.2
Accuracy (%)		84.4	81.3	75.0	39.1	95.3	87.5

AUC, area under the receiver operating characteristic (ROC) curve; SEM, standard error; asterisk (*) indicated the cut-off value (OD) recommended by the manufacturer.

blocked plates with different storage temperatures (RT, 4°C, −30°C) as well as different storage time (from day 0 to day 60) on Rapid-LACA results, the data obtained from each assay plate were compared to each other and expressed as the ratio of luciferase activity of positives to negatives (P/N ratio) (Fig. 5). The results indicated that stored plates at different temperatures could be used in Rapid-LACA over time with a high consistency with each other. Even though the P/N ratios obtained at each time points might be variable, the differences were not significant and the assay was still able to unequivocally discriminate between the positive and negative signals during different storage periods.

DISCUSSION

In the present study, we validated the sensitivity of LACA more reliably when employing a set of serum samples from experimentally *T. gondii* infected and uninfected pigs as true positives and true negatives (Fig. 1, Table 1). To evaluate the specificity of LACA, we used the set of experimentally infected pig sera with *A. suum* and *T. cati*, which are well known as the common roundworms found in pigs and cats, respectively. As previously reported, pigs are listed as a suitable paratenic host for

Table 2. Kappa coefficient values of different rNluc-fused *Toxoplasma gondii* antigens in Luciferase-linked Antibody Capture Assay (LACA) compared to Dye-Test as "gold standard" using pig serum samples

		Dye-Test		Kappa value	95% CI
		Positive	Negative		
GRA6-LACA	Positive	13	25	0.230	0.044 to 0.417
	Negative	8	53		
GRA7-LACA	Positive	10	7	0.415	0.192 to 0.638
	Negative	11	71		
GRA8-LACA	Positive	17	24	0.372	0.199 to 0.545
	Negative	4	54		
BAG1-LACA	Positive	4	11	0.055	–0.151 to 0.261
	Negative	17	67		
Cocktail LACA	Positive	21	7	0.811	0.679 to 0.944
	Negative	0	71		
Commercial ELISA	Positive	2	6	0.024	–0.155 to 0.203
	Negative	19	72		
	Total	21	78		

CI, confidence interval.

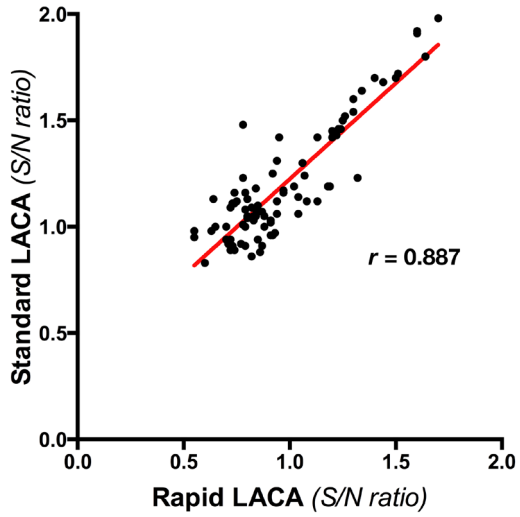

Fig. 4. Correlation plot comparing standard Luciferase-linked Antibody Capture Assay (LACA) and Rapid-LACA employing the set of individual pig serum samples. The correlation was assessed by Spearman analyses with listed r-value and linear regression. The graph displays the correlation between Rapid-LACA and standard LACA. The values obtained from each test are expressed by S/N ratio. Black dots represent individual pig serum samples (n=90).

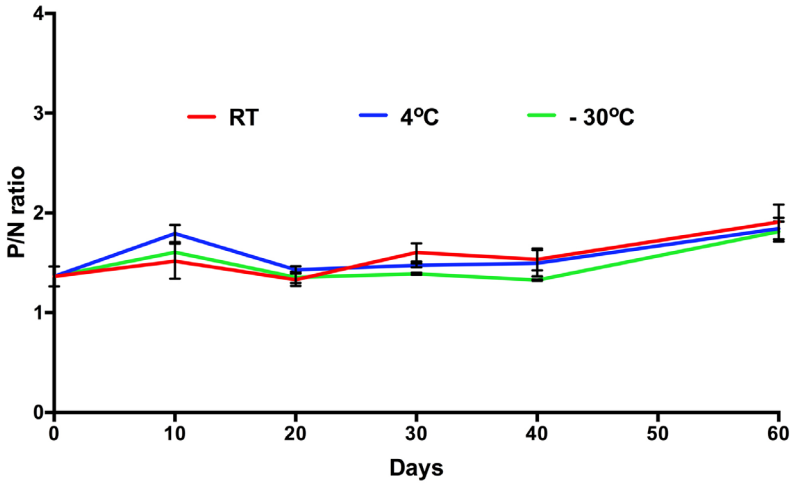

Fig. 5. Long-term stability of storaged protein A pre-coated/blocked plates at different temperatures. Protein A pre-coated/blocked plates were stored at room temperature (RT), 4°C and −30°C until the time of analysis. The P/N ratios were calculated from the mean value of ratio between luciferase activity of positive sera and the luciferase activity of negative sera. For each storage time point and temperature, the mean value of P/N ratio is shown.

this feline roundworm species [7, 23]. Therefore, cats are the source of both *T. gondii* and *T. cati* infection for pigs on farms. Unfortunately, cross-reactivity with antibodies for related parasites, such as *Cystoisospora* spp. and *Sarcocystis* spp., could not be investigated due to the limitation of positive sera infected with these protozoa experimentally. The prevalence of *Cystoisospora* spp. and *Sarcocystis* spp. in pigs has been reported to be 12.0–17.0% and 8.0–9.0% in Japan, respectivelly [32, 33]. Further study is needed to assess diagnostic characteristics of our LACA. From our results, the combination of rNluc-GRA6, rNluc-GRA7 and rNluc-GRA8 antigens in cocktail LACA exhibited the best diagnostic performance compared to LACA using separately single rNluc-GRA6, rNluc-GRA7 or rNluc-GRA8 antigen as well as commercial ELISA. Conversely, rNluc-BAG1 showed fairly weak immunoreactivity and had no discrimination capacity to distinguish between positive and negative signals. BAG1 has been commonly referred to as a specific indicator for *T. gondii* bradyzoite in *in-vitro* and *in-vivo* experiments because the BAG1 gene

was upregulated during the stage differentiation and BAG1 antigen was found abundantly in the cytoplasm of bradyzoites [9, 34, 42]. Nevertheless, the relatively weak immunogenicity of BAG1 antigen that we observed in this study was in agreement with results obtained from studies by Di Cristina (2004), Dautu (2007) and Nau (2017) [6, 10, 28]. Meanwhile, dense granule proteins GRAs abundantly secreted by both *T. gondii* tachyzoites and bradyzoites exhibit strong immunogenicity. These immunogenic antigens could be detected throughout the *T. gondii* infection process at both acute and chronic phases [20, 30]. Hence, the prolonged expression of GRAs as well as their immunogenicity make them ideal diagnostic antigens [43]. Among them, GRA1, GRA2, GRA3, GRA4, GRA5, GRA6, GRA7, and GRA8 have been evaluated for their potential as molecular markers with high sensitivities for the detection of anti-*Toxoplasma* antibodies [43]. Particularly, GRA7 has been well recognized for its excellent diagnostic ability for *Toxoplasma* infection in pigs [40].

Furthermore, it has been reported that the combination of several antigens as mixtures provided an improvement in ELISA for human toxoplasmosis compared to each individual antigen, such as a combination of GRA7, GRA8 and Rhoptry 1, of GRA7, GRA8 and SAG1, or of GRA6, GRA8 and SAG2 [18]. In this study, the rNluc-BAG1 showed poor diagnostic performance [25], therefore it was not used in cocktail antigen. Meanwhile, combining rNluc-GRA6, rNluc-GRA7 and rNluc-GRA8 together improved the sensitivity and specificity of LACA (Fig. 2, Table 1). It is likely that the obtained luminescent signal in LACA is proportional to the number of recombinant nanoluciferase fussion antigens bound to target antibodies. Therefore, antigen mixture could increase the number of bindings, thereby increasing the ability for signal detection and widening dynamic range that could lead to both increased sensitivity and specificity in cocktail LACA when compared with the use of a single antigen. Additionally, the strong agreement of cocktail LACA to "gold standard" Dye-Test in the pig serum samples collected from the field indicated that the cocktail LACA could be a reliable and applicable serodiagnostic test for *T. gondii* infection in pigs (Table 2).

In addition, we also successfully developed and optimized the new format of LACA as Rapid-LACA which showed promising advantages in practical applications (Fig. 4). Compared to standard LACA or conventional ELISA which have many steps of incubation and washing and usually takes approximately 3–4 hr to complete, Rapid-LACA with single co-incubation of sera and antigen mixture could be done within 30 min. For Rapid LACA designed for pigs, we used protein A pre-coated/blocked plates. Protein A here was used to capture IgG in examined pig serum samples. We assessed the effect of temperature and storage time on long-term preserved protein A pre-coated/blocked plates. Unexpectedly, the coated plates could be kept at least 2 months even at RT without any loss in their efficiency (Fig. 5). Our results indicated that the standard LACA could be replaced by the Rapid-LACA with more convenient and simple procedure. Additionally, the storage temperature for protein A pre-coated/blocked plates had no effect on the results of the tests. Instead of preparing fresh LACA plates each time when needed, the stock of pre-coated/blocked plates used in Rapid-LACA would be more time efficient.

Besides, the antigenic strain variation in *T. gondii* has been previously reported [1, 41]. However, the employment of antigens from different *Toxoplasma* strains might not affect the serological diagnosis because of common antigenic epitopes [8]. To confirm the applicability of our system for the diagnosis of *Toxoplasma* infection caused by different *Toxoplasma* strains or genotypes, further studies should be addressed.

Altogether, the antigen mixture of rNluc-GRA6, rNluc-GRA7 and rNluc-GRA8 could improve the diagnostic accuracy of LACA for the detection of *T. gondii* infection in pigs. The novel Rapid-LACA format represents a simple diagnostic test that is effectively time-saving and easy to perform.

CONFLICT OF INTEREST. The authors declare that they have no conflict of interest.

ACKNOWLEDGMENTS. This work was supported by grants from the Ministry of Health and the Ministry of Education, Culture, Sports, Science and Technology (Special Education and Research Expenses "Establishment of world-wide advanced research and medical network in HTLV-1/ATL"), Japan Science and Technology Agency for Science and Technology Research Partnership for Sustainable Development (SATREPS) (JPMJSA1908) and University of Miyazaki (2020 Support Program for Young Researchers).

REFERENCES

1. Arranz-Solís, D., Carvalheiro, C. G., Zhang, E. R., Grigg, M. E. and Saeij, J. P. J. 2021. *Toxoplasma* GRA Peptide-specific serologic fingerprints discriminate among major strains causing toxoplasmosis. *Front. Cell. Infect. Microbiol.* **11**: 621738. [Medline] [CrossRef]
2. Aye, K. M., Nagayasu, E., Baba, M., Yoshida, A., Takashima, Y. and Maruyama, H. 2018. Evaluation of LIPS (luciferase immunoprecipitation system) for serodiagnosis of Toxoplasmosis. *J. Immunol. Methods* **462**: 91–100. [Medline] [CrossRef]
3. Basso, W., Hartnack, S., Pardini, L., Maksimov, P., Koudela, B., Venturini, M. C., Schares, G., Sidler, X., Lewis, F. I. and Deplazes, P. 2013. Assessment of diagnostic accuracy of a commercial ELISA for the detection of *Toxoplasma gondii* infection in pigs compared with IFAT, TgSAG1-ELISA and Western blot, using a Bayesian latent class approach. *Int. J. Parasitol.* **43**: 565–570. [Medline] [CrossRef]
4. Cubas-Atienzar, A. I., Hide, G. and Smith, J. E. 2019. Mat Seroprevalence infers low rates of *Toxoplasma gondii* in domestic pigs from Yucatan, Mexico. *J. Parasitol.* **105**: 738–747. [Medline] [Crossref]
5. Dannemann, B. R., Vaughan, W. C., Thulliez, P. and Remington, J. S. 1990. Differential agglutination test for diagnosis of recently acquired infection with *Toxoplasma gondii*. *J. Clin. Microbiol.* **28**: 1928–1933. [Medline] [CrossRef]
6. Dautu, G., Munyaka, B., Carmen, G., Zhang, G., Omata, Y., Xuenan, X. and Igarashi, M. 2007. *Toxoplasma gondii*: DNA vaccination with genes encoding antigens MIC2, M2AP, AMA1 and BAG1 and evaluation of their immunogenic potential. *Exp. Parasitol.* **116**: 273–282. [Medline] [CrossRef]
7. Davidson, R. K., Mermer, A. and Øines, Ø. 2012. *Toxocara cati* larva migrans in domestic pigs-detected at slaughterhouse control in Norway. *Acta Vet. Scand.* **54**: 66. [Medline] [CrossRef]

8. Delibas, S. B., Ertabaklar, H. and Ertug, S. 2006. Evaluation of antigenic variations between two virulent toxoplasma strains. *J. Med. Microbiol.* **55**: 1333–1335. [Medline] [CrossRef]
9. Di Cristina, M., Marocco, D., Galizi, R., Proietti, C., Spaccapelo, R. and Crisanti, A. 2008. Temporal and spatial distribution of *Toxoplasma gondii* differentiation into Bradyzoites and tissue cyst formation *in vivo*. *Infect. Immun.* **76**: 3491–3501. [Medline] [CrossRef]
10. Di Cristina, M., Del Porto, P., Buffolano, W., Beghetto, E., Spadoni, A., Guglietta, S., Piccolella, E., Felici, F. and Gargano, N. 2004. The *Toxoplasma gondii* bradyzoite antigens BAG1 and MAG1 induce early humoral and cell-mediated immune responses upon human infection. *Microbes Infect.* **6**: 164–171. [Medline] [CrossRef]
11. Dubey, J. P. 2009. Toxoplasmosis in pigs—the last 20 years. *Vet. Parasitol.* **164**: 89–103. [Medline] [CrossRef]
12. Dubey, J. P., Thulliez, P., Weigel, R. M., Andrews, C. D., Lind, P. and Powell, E. C. 1995. Sensitivity and specificity of various serologic tests for detection of *Toxoplasma gondii* infection in naturally infected sows. *Am. J. Vet. Res.* **56**: 1030–1036. [Medline]
13. Duong, H. D., Appiah-Kwarteng, C., Takashima, Y., Aye, K. M., Nagayasu, E. and Yoshida, A. 2020. A novel luciferase-linked antibody capture assay (LACA) for the diagnosis of *Toxoplasma gondii* infection in chickens. *Parasitol. Int.* **77**: 102125. [Medline] [CrossRef]
14. Ferra, B., Holec-Gąsior, L. and Grążlewska, W. 2020. *Toxoplasma gondii* recombinant antigens in the Serodiagnosis of Toxoplasmosis in domestic and farm animals. *Animals (Basel)* **10**: 1245. [Medline]
15. Foroutan, M., Fakhri, Y., Riahi, S. M., Ebrahimpour, S., Namroodi, S., Taghipour, A., Spotin, A., Gamble, H. R. and Rostami, A. 2019. The global seroprevalence of *Toxoplasma gondii* in pigs: a systematic review and meta-analysis. *Vet. Parasitol.* **269**: 42–52. [Medline] [CrossRef]
16. Gisev, N., Bell, J. S. and Chen, T. F. 2013. Interrater agreement and interrater reliability: key concepts, approaches, and applications. *Res. Social Adm. Pharm.* **9**: 330–338. [Medline] [CrossRef]
17. Hagiwara, T. and Katsube, Y. 1981. Detection of *Toxoplasma* infectionin pork by Sabin-Feldman's dye test with meat extract. *Nippon Juigaku Zasshi* **43**: 763–765. [Medline] [CrossRef]
18. Holec-Gąsior, L. 2013. *Toxoplasma gondii* recombinant antigens as tools for serodiagnosis of human toxoplasmosis: current status of studies. *Clin. Vaccine Immunol.* **20**: 1343–1351. [Medline] [CrossRef]
19. Hoshina, T., Fukumoto, S., Aonuma, H., Saiki, E., Hori, S. and Kanuka, H. 2019. Seroprevalence of *Toxoplasma gondii* in wild sika deer in Japan. *Parasitol. Int.* **71**: 76–79. [Medline] [CrossRef]
20. Hughes, H. P. and van Knapen, F. 1982. Characterisation of a secretory antigen from *Toxoplasma gondii* and its role in circulating antigen production. *Int. J. Parasitol.* **12**: 433–437. [Medline] [CrossRef]
21. Kijlstra, A. and Jongert, E. 2008. Control of the risk of human toxoplasmosis transmitted by meat. *Int. J. Parasitol.* **38**: 1359–1370. [Medline] [CrossRef]
22. Koike, N., Mai, T. N., Shirai, M., Kubo, M., Hata, K., Marumoto, N., Watanabe, S., Sasaki, Y., Mitoma, S., Notsu, K., Okabayashi, T., Wiratsudakul, A., Kabali, E., Norimine, J. and Sekiguchi, S. 2018. Detection of neutralizing antibody against porcine epidemic diarrhea virus in subclinically infected finishing pigs. *J. Vet. Med. Sci.* **80**: 1782–1786. [Medline] [CrossRef]
23. Křivanec, K. 1980. The finding of eggs of *Toxocara cati* in droppings of fattened pigs. *J. Parasitol.* **66**: 168–169. [Medline] [CrossRef]
24. Li, J., Wang, G., Yang, D., Zhao, B., Zhao, Y., Liu, Y., Cai, X., Nan, Y., Zhou, E. M. and Wu, C. 2018. Development of luciferase-linked antibody capture assay based on luciferase immunoprecipitation systems for antibody detection of porcine reproductive and respiratory syndrome virus. *BMC Biotechnol.* **18**: 73. [Medline] [CrossRef]
25. Mandrekar, J. N. 2010. Receiver operating characteristic curve in diagnostic test assessment. *J. Thorac. Oncol.* **5**: 1315–1316. [Medline] [CrossRef]
26. Mukaka, M. M. 2012. Statistics corner: a guide to appropriate use of correlation coefficient in medical research. *Malawi Med. J.* **24**: 69–71. [Medline]
27. Nam, H. W. 2009. GRA proteins of *Toxoplasma gondii*: maintenance of host-parasite interactions across the parasitophorous vacuolar membrane. *Korean J. Parasitol.* **47** Suppl: S29–S37. [Medline] [CrossRef]
28. Nau, J., Eller, S. K., Wenning, J., Spekker-Bosker, K. H., Schroten, H., Schwerk, C., Hotop, A., Groß, U. and Däubener, W. 2017. Experimental Porcine *Toxoplasma gondii* Infection as a representative model for human Toxoplasmosis. *Mediators Inflamm.* **2017**: 3260289. [Medline] [CrossRef]
29. Reiter-Owona, I., Petersen, E., Joynson, D., Aspöck, H., Dardé, M. L., Disko, R., Dreazen, O., Dumon, H., Grillo, R., Gross, U., Hayde, M., Holliman, R., Ho-Yen, D. O., Janitschke, K., Jenum, P. A., Naser, K., Olszewski, M., Thulliez, P. and Seitz, H. M. 1999. The past and present role of the Sabin-Feldman dye test in the serodiagnosis of toxoplasmosis. *Bull. World Health Organ.* **77**: 929–935. [Medline]
30. Rezaei, F., Sharif, M., Sarvi, S., Hejazi, S. H., Aghayan, S., Pagheh, A. S., Dodangeh, S. and Daryani, A. 2019. A systematic review on the role of GRA proteins of *Toxoplasma gondii* in host immunization. *J. Microbiol. Methods* **165**: 105696. [Medline] [CrossRef]
31. Sabin, A. B. and Feldman, H. A. 1948. Dyes as Microchemical Indicators of a New Immunity Phenomenon Affecting a Protozoon Parasite (Toxoplasma). *Science* **108**: 660–663. [Medline] [CrossRef]
32. Saito, M., Nakajima, T., Watanabe, A. and Itagaki, H. 1986. *Sarcocystis miescheriana* infection and its frequency in pigs in Japan. *Nippon Juigaku Zasshi* **48**: 1083–1090. [Medline] [CrossRef]
33. Saitoh, Y. and Hattori, J. 2007. Survey of porcine neonatal coccidium *Isospora suis* (Biester and Murray 1934) in Japan. *Proc. Japan Pig Vet. Soc.* **50**: 7–8.
34. Soête, M., Camus, D. and Dubremetz, J. F. 1994. Experimental induction of bradyzoite-specific antigen expression and cyst formation by the RH strain of *Toxoplasma gondii in vitro*. *Exp. Parasitol.* **78**: 361–370. [Medline] [CrossRef]
35. Sroka, J., Cencek, T., Ziomko, J. and Karamon, J. 2008. Preliminary assessment of ELISA, MAT, and LAT for detecting *Toxoplasma gondii* antibodies in pigs. *Bull. Vet. Inst. Pulawy.* **52**: 545–549
36. Steinparzer, R., Reisp, K., Grünberger, B., Köfer, J., Schmoll, F. and Sattler, T. 2015. Comparison of different commercial serological tests for the detection of *Toxoplasma gondii* antibodies in serum of naturally exposed pigs. *Zoonoses Public Health* **62**: 119–124. [Medline] [CrossRef]
37. Taniguchi, Y., Appiah-Kwarteng, C., Murakami, M., Fukumoto, J., Nagamune, K., Matsuo, T., Masatani, T., Kanuka, H., Hoshina, T., Kitoh, K. and Takashima, Y. 2018. Atypical virulence in a type III *Toxoplasma gondii* strain isolated in Japan. *Parasitol. Int.* **67**: 587–592. [Medline] [CrossRef]
38. Taniguchi, Y., Yanagihara, I., Nakura, Y., Ichikawa, C., Saito, T., Appiah-Kwarteng, C., Matsuzaki, M., Fukumoto, J., Nagamune, K., Kyan, H., Takasu, M., Kitoh, K. and Takashima, Y. 2019. A *Toxoplasma gondii* strain isolated in Okinawa, Japan shows high virulence in Microminipigs. *Parasitol. Int.* **72**: 101935. [Medline] [CrossRef]
39. Tenter, A. M., Heckeroth, A. R. and Weiss, L. M. 2000. *Toxoplasma gondii*: from animals to humans. *Int. J. Parasitol.* **30**: 1217–1258. [Medline] [CrossRef]
40. Terkawi, M. A., Kameyama, K., Rasul, N. H., Xuan, X. and 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. [Medline] [CrossRef]
41. Ware, P. L. and Kasper, L. H. 1987. Strain-specific antigens of *Toxoplasma gondii*. *Infect. Immun.* **55**: 778–783. [Medline] [CrossRef]
42. Weiss, L. M. and Kim, K. 2000. The development and biology of bradyzoites of *Toxoplasma gondii*. *Front. Biosci.* **5**: D391–D405. [Medline] [CrossRef]
43. Ybañez, R. H. D., Ybañez, A. P. and Nishikawa, Y. 2020. Review on the current trends of Toxoplasmosis Serodiagnosis in humans. *Front. Cell. Infect. Microbiol.* **10**: 204. [Medline] [CrossRef]