Seroepidemiological study of *Toxoplasma* infection in the Philippines and the application of an immunochromatographic test for its diagnosis

2020

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フィリピンにおける
トキソプラズマ感染の血清疫学調査と
イムノクロマトテストの診断への応用

令和 2 年

(2020)

帯広畜産大学大学院畜産学研究科

博士後期課程　畜産衛生学専攻

イバニエズ　ロシェル　ハイデイー　ダクラン

Contents

List of figures

List of tables

Abbreviations and unit abbreviations

Abbreviations

A.	AIDS	: Acquired immune deficiency syndrome
B.	BCA	: Bicinchoninic acid
C.	cDNA	: Complementary DNA
	CI	: Confidence interval
D.	DAT	: Direct agglutination test
E.	ELISA	: Enzyme-linked immunosorbent assay
	EMEM	: Eagle's minimum essential medium
F.	FBS	: Fetal bovine serum
G.	GST	: Glutathione S-transferase
H.	HIV	: Human immunodeficiency virus
	HRP	: Horseradish peroxidase
I.	ICT	: Immunochromatographic test
	iELISA	: Indirect enzyme-linked immunosorbent assay
	IFA	: Indirect fluorescent assay
	IFAT	: Immunofluorescent antibody test
	IgA	: Immunoglobulin A

	IgE	: Immunoglobulin e
	IgG	: Immunoglobulin G
	IgM	: Immunoglobulin M
	IHAT	: Indirect hemagglutination test
L.	LAT	: Latex agglutination test
M.	MAT	: Modified agglutination test
O.	OD	: Optical density
	OR	: Odds ratio
P.	PBS	: Phosphate-buffered saline
	PCR	: Polymerase chain reaction
	Pearson's r	: Pearson correlation coefficient
	pH	: Power of hydrogen
	PLK	: *Toxoplasma gondii* type II representative strain
	PV	: Parasitophorous vacuole
	PVM	: Parasitophorous vacuole membrane
	P-value	: Probability value
R.	RBC	: Red blood cells
	RH	: *Toxoplasma gondii* type I representative strain
	RNA	: Ribonucleic acid
	rTgGRA7	: Recombinant *Toxoplasma gondii* dense granule 7
S.	SAG2	: Surface antigen 2

	SDS-PAGE	: Sodium dodecyl sulfate polyacrylamide gel Electrophoresis
	SFDT	: Sabin-Feldman dye test
T.	*T. gondii*	: *Toxoplasma gondii*
	TgGRA	: *Toxoplasma gondii* dense granule antigen
	TgGRA7	: *Toxoplasma gondii* dense granule 7
	TgMAG1	: *Toxoplasma gondii* matrix antigen 1
	TgROP1	: *Toxoplasma gondii* rhoptry 1
	TgSAG1	: *Toxoplasma gondii* surface antigen 1
	TLA	: Tachyzoite lysate antigen
V.	VEG	: *Toxoplasma gondii* type III representative strain

Unit abbreviations

°C	: degree Celsius
hr	: hour
IU	: international units
kDa	: kilodalton
kHZ	: kilohertz
µg	: microgram
µL	: microliter
µm	: micrometer

μM	: micromolar
mg	: milligram
mL	: milliliter
mm	: millimeter
mM	: millimolar
min	: minute
nm	: nanometer
%	: percentage
sec	: second

General introduction

1. Toxoplasmosis

Toxoplasmosis is a widely distributed zoonotic infection caused by the obligate intracellular apicomplexan parasite, *Toxoplasma gondii*. *T. gondii* infects humans and almost all warm-blooded animals, making it one of the important parasites affecting public health and animal production. It is mainly transmitted through the ingestion of oocysts shed by an infected cat as its definitive host (Dubey and Beattie, 1988; Dubey and Jones, 2008; Torrey and Yolken, 2013). Toxoplasmosis affects approximately one-third of the world's human population. However, it is generally asymptomatic in immunocompetent individuals, or it may manifest flu-like symptoms and other non-specific clinical signs (Dubey, 1991). The disease may even be severe or fatal in immunocompromised patients (Montoya and Liesenfeld, 2004). Vertical transmission of the parasite through the placenta from the infected mother may compromise the life of the fetus and the infected mothers (Gatkowska et al., 2006; Elmore and Jones, 2010). The key to effective control and treatment of toxoplasmosis depends on accurate detection of *T. gondii* infection. The utilization of highly sensitive and specific diagnostic methods is a vital step in the prevention and treatment of the disease (Liu et al., 2015; Pomares and Montoya, 2016).

Due to its non-specificity of clinic signs, the diagnosis of *T. gondii* infection cannot be made through the assessment of clinical manifestations (Tenter et al., 2000). *T. gondii* diagnosis for immunocompromised patients is usually done using polymerase chain reaction (PCR), hybridization assays, isolation, and histological analysis. For congenital cases,

diagnosis is through direct detection of the organism through mouse inoculation, cell culture or PCR from samples collected from amniotic fluid (Cazenave et al., 1991), cerebrospinal fluid, blood and urine (Fuentes et al., 1996), and through ophthalmologic and radiological examinations (Montoya, 2002; Pomares and Montoya, 2016). However, the most common form of *T. gondii* infection is latent, wherein the parasites are usually not found in the circulation, and isolating the parasites are particularly challenging (Robert-Gangneux and Dardé, 2012). Moreover, *T. gondii*-infected individuals were observed to elicit high IgG antibody titers regardless of the clinical presentation. These marked antibody titers, which were also detectable over a long period, are evidence of a robust and lasting humoral immune response against the infection (Parmley et al., 1992; Dubey, 2008, Maksimov et al., 2013). Thus, serological tests that detect specific antibody responses are deemed useful.

2. Serodiagnosis of toxoplasmosis

As a direct demonstration of the *T. gondii* parasite is often difficult, several serodiagnostic methods have been developed. These methods, which detect different antibodies (Montoya, 2002; Sudan et al., 2013) or antigens (Desmonts et al., 1981), have been used to achieve reliable diagnosis. In most epidemiological studies of toxoplasmosis, serological tests have been mainly preferred (Montoya, 2002; Robert-Gangneux and Dardé, 2012) and appear to be the primary approach in satisfactorily evaluating disease investigations (Rorman et al., 2006). The generation of each antibody isotype is directly related to the humoral immunity following infection. Hence, by monitoring these responses, it can be determined whether or not the host has *Toxoplasma* infection. Due to the non-specificity of

clinical signs of toxoplasmosis, serological test results have been paired with clinical signs evaluation in diagnosing toxoplasmosis (Montoya, 2002; Lopes et al., 2007).

The levels of different types of antibodies, including IgM, IgG, IgA, and IgE, are measured by the tests, which increases and decreases during or after infection (Rorman et al., 2006; Dubey, 2008). IgM is serologically detected one week after infection, and therefore, is regarded as a prompt and sensitive marker for acute toxoplasmosis diagnosis. However, it may also be serologically present for several months or years (Liu et al., 2015). In an infected pregnancy, IgM antibodies can be detected in the maternal circulation even 18 months after infection and may confuse interpretation whether the detected antibody is from active or previous infection (Bortoletti Filho et al., 2013). If the antibody is from a prior infection, there are no consequences usually observed in the fetus. However, if the infection happens during pregnancy, the clinicians must administer anti - parasitic treatment to avoid disease complications in the unborn child (Montoya, 2002; Lopes et al., 2007). Interpretation of results based on IgM levels can, therefore, be sometimes tricky and insufficient (Liu et al., 2015).

IgG antibodies against *T. gondii* can be detected 1–2 weeks following infection. It peaks typically within 1–2 months and declines at different rates. As it can persist permanently at lingering titers, this antibody is an indicator of a previous infection. It has since been used as a standard diagnostic marker for chronic infection. However, this antibody still struggles in differentiating past and present infections. An ancillary IgG based-test has been developed to distinguish acute from chronic infection in an asymptomatic individual (Montoya, 2002; Lopes et al., 2007). Other tests based on IgE and IgA have been developed. These antibodies are made during the first weeks of infection and wane early (Robert - Gangneux and Dardé, 2012). Various serological procedures have already been established to determine recent and previous

exposures: Sabin-Feldman dye test (SFDT) (Sabin and Feldman, 1948), agglutination tests (Dubey, 1997; Dubey, 2008; Robert-Gangneux and Dardé, 2012; Liu et al., 2015), indirect fluorescent assay (IFA) (Rorman et al., 2006; Saraei et al., 2010), and enzyme-linked immunosorbent assays (ELISAs) (Voller et al., 1976; Redlich and Müller, 1998; Pietkiewicz et al., 2004; Ferra et al., 2019), or a combination of these methods (Rorman et al., 2006; Dubey, 2008; Robert-Gangneux and Dardé, 2012).

2.1. Sabin-Feldman dye test (SFDT)

The Sabin-Feldman dye test (SFDT) was developed more than seven decades ago (Sabin and Feldman, 1948) for the investigation of *T. gondii* infection in the laboratory (Rorman et al., 2006). SFDT has high sensitivity and specificity and is still considered as the "gold standard" (Reiter-Owona et al., 1999). It utilizes complementation of live tachyzoite incubation with patient serum. If the serum has *T. gondii*-specific antibodies, the parasites will be subsequently coated and lysed by the complement system, and staining with methylene blue dye will not happen. The number of stained (live) and unstained (dead) tachyzoites are counted to determine the end-point titer (Reiter-Owona et al., 1999; Rorman et al., 2006; Udonsom et al., 2010). While SFDT can detect both IgM and IgG, the antibody titers cannot accurately differentiate between acute or chronic infection. Moreover, SFDT entails using live parasites, which is a biohazard, thereby limiting its application to only a few laboratories (Reiter-Owona et al., 1999; Udonsom et al., 2010).

2.2. Agglutination tests

Agglutination tests require particulate antigens that can bind with antibodies. Multivalent antibodies (called agglutinins) form large clumps or aggregates with suspended particulate antigens when present, which can be visually seen without magnification. These tests are used to determine concentrations of specific antibodies. In toxoplasmosis diagnosis in humans and animals, different agglutination tests, including direct agglutination test (DAT), modified agglutination test (MAT), indirect hemagglutination test (IHAT), and latex agglutination test (LAT), have been used (Dubey, 1997; Dubey, 2008; Robert-Gangneux and Dardé, 2012; Liu et al., 2015). DAT was developed in 1965 and has since been very useful in detecting anti-*T. gondii* antibodies in humans and animals (Dubey, 2008). But it is utilized for the detection of IgG antibodies only. It is very simple as it does not require a secondary antibody and specialized equipment. In DAT, diluted patient sera are added to microtiter plates that are coated with formalinized *Toxoplasma* tachyzoites. Subsequent agglutination happens if antibodies against *Toxoplasma* are present in the sera. If the sample is negative, tachyzoites will be precipitated at the bottom of the wells (Desmonts and Remington, 1980). While DAT is considered very sensitive and economical, it requires a large antigen amount. Moreover, the presence of IgM antibodies in the sera causes non-specific agglutination (Dubey, 2008). The MAT is an adaptation of the DAT with some adjustments involving the preparation of the antigen and incubation period of the test plates (Dubey, 1997; Al-Adhami et al., 2016).

IHAT employes red blood cells (RBCs) that are sensitized with a soluble antigen of *T. gondii*. The sensitized cells will subsequently clump together if the sera have anti-*T. gondii* antibodies. IHAT is also considered very simple and economical (Liu et al., 2015). On the other hand, LAT utilizes covalently bonded tachyzoite particles that are coated to latex beads.

A visible agglutination reaction is observed when the sera contain specific IgG antibody (Mazumder et al., 1988). The sensitivity and specificity of LAT in humans and animals range from low to high. Same with other agglutination tests, non-specific IgM agglutinations can also happen in LAT which can generate false-positive results (Ohshima et al., 1981; Mazumder et al., 1988; Oncel et al., 2005; Robert-Gangneux and Dardé, 2012). LAT has been used often as a screening test for epidemiological studies before other serological tests are utilized for further examination (Holliman et al., 1990).

2.3. Indirect fluorescent assay (IFA)

IFA is an alternative simple and safe diagnostic method that does not use live tachyzoites (Rorman et al., 2006; Saraei et al., 2010). This assay is based on the specific antigen-antibody interaction from diluted serum specimens with killed *Toxoplasma* tachyzoites. The reaction will then be identified by adding fluorescent-labeled anti-human IgG or IgM antibodies under a fluorescence microscope (Pappas et al., 1986). Among the limitations of IFA include the individual differences in result reading and the probability of false-positive findings in case rheumatoid factors or antinuclear antibodies are present in the sera (Rorman et al., 2006). Nonetheless, high *T. gondii*-specific IgG titers in some newly acquired toxoplasmosis patients may impede the IgM antibodies and cause false-negative results (Remington et al., 1985).

2.4. Enzyme-linked immunosorbent assay (ELISA)

Even after four decades since it was established in toxoplasmosis diagnosis (Voller et al., 1976), ELISA is still considered one of the most common techniques with high sensitivity and specificity in the quantitative detection of antibodies and all antigenically active molecules (Döskaya et al., 2014; Liu et al., 2015). The ELISA system typically consists of an antigen or antibody immobilized on a solid phase or microtiter plate, an enzyme-conjugated antigen or antibody, and an enzymatic substrate, which can be adjusted to analyze either antibodies or antigens (Liu et al., 2015). There are different kinds of ELISA developed to detect *T. gondii* antibodies or antigens, namely indirect ELISA, sandwich ELISA, and dot-ELISA. The indirect ELISA (iELISA) involves coating a microtiter plate with antigens and the application of sera, which contains antibodies. The presence of anti-*Toxoplasma* antibodies leads to consequent binding with the coated antigen and is detected by using an enzyme-conjugated secondary antibody. The subsequent washing steps will remove any unbound reagents, and when the substrate is finally added, color reaction develops. This type of ELISA is mostly used to detect IgM, IgG, and IgA antibodies against *T. gondii* instead of antigens (Tomasi et al., 1986). The standard iELISAs utilizing *T. gondii* lysate antigen (TLA) revealed a high level of concordance with SFDT, IFAT or MAT for the detection of IgM or IgG antibodies in humans and animals (Filice et al., 1983; Tomasi et al., 1986; Obwaller et al., 1995). In the sandwich ELISA, the microtiter plate is coated with capture antibodies, and serum samples containing *T. gondii* antigens is added. The plate is incubated and washed, and the enzyme-conjugated secondary antibody is added to each well to detect the capture antibody-antigen reaction. Following subsequent washings, the substrate is added for a color reaction to develop. Compared to IFAT, the sandwich ELISA using TLA is reported to be more sensitive and specific to detect human

IgM (Tomasi et al., 1986). The dot-ELISA is a modified ELISA where the antigen-antibody reaction is done on nitrocellulose instead of the microtiter plate. This test is sensitive to detect *T. gondii* antigens and antibodies (Pappas et al., 1986; Jafar Pour Azami et al., 2011) and does not require any special equipment, thus easier to perform than standard ELISAs (Pappas et al., 1986; Youssef et al., 1992). The quantity of antibodies detected in the sera using ELISA is shown to be positively correlated with the intensity of the color reaction. Result interpretation usually is dependent on the qualitative assessment of color change spectrophotometrically. Deciding samples to be positive or negative is achieved by comparing the optical density of the sera with the control after setting a threshold value (Seefeldt et al., 1989).

The ELISA is primarily utilized for routine screening of *T. gondii* infections because it is highly sensitive (allowing quantitative and semi-quantitative antibody assessments), easily implemented, and low-cost (Shaapan et al., 2008). It can be simply used to test large populations in a short period of time (Sudan et al., 2013), with the capability to detect both IgG and IgM (Seefeldt et al., 1989). This method is also primarily used to evaluate the efficacy of different recombinant proteins as antigens for serodiagnosis. However, standardization of used antigens in ELISA has been challenging (Shaapan et al., 2008). In cases of a weak positive reaction, a photometer is required to differentiate it from a negative reaction, thereby increasing the cost (Seefeldt et al., 1989). False-positive results can also happen in IgM-based ELISA (Fuccillo et al., 1986; Liesenfeld et al., 1997), possibly due to rheumatoid factors in the serum, while IgG-based ELISA can result in false-negative results probably owing to competitive inhibition of specific IgG (Fuccillo et al., 1986). The low-level IgG detection is a problem, i.e. IgG results from the "gray zone" in ELISA. According to Robert-Gangneux and Dardé (2012), these low titers must be confirmed by using a dye test or a sensitive Western blot (WB) assay.

2.5. Immunochromatographic tests (ICT)

ICT is a rapid lateral flow test intended to detect the presence or absence of the target analyte. The principle of ICT is based on a dye-labeled antibody or colloidal gold-labeled antigen that is specific for the target analyte in the liquid sample, which is present on the lower end of the nitrocellulose strip or in the plastic well along with the strip. The sample is dropped at the designated part on the nitrocellulose membrane, which will gradually permeate the gold colloid-conjugated pad via capillary flow, and consequent antigen-antibody complex will demonstrate color reaction (Wang et al., 2011). It is simple because specialized and costly equipment may not be needed, although several laboratory-based applications and reading equipment may exist. It is believed to be a low-cost test which facilitates the rapid identification of analytes at the point of care (Weiss, 1999; Zhang et al., 2009; Thobhani, et al., 2010; Goni et al., 2012; Yetisen et al., 2013). Its ease of application and rapidity of test results with no special equipment required makes the ICT suitable for field application. In toxoplasmosis, this technology has been used to diagnose human (Lévêque et al., 2019; Taha et al., 2019; Wassef and Abdel-Malek, 2019) and animal cases (Khan and Noordin, 2019). ICT has been demonstrated to be in high concordance with ELISA with regard to sensitivity and specificity (Terkawi et al., 2013; Ybañez and Nishikawa, 2020; Ybañez et al., 2020). ICT based on GRA7 (Terkawi at el., 2013; Ybañez and Nishikawa, 2020; Ybañez et al., 2020) and SAG2 (Huang et al., 2004) show high consistency with results obtained from LAT and ELISA.

2.6. Western blotting

The Western blot (sometimes referred to as immunoblot) aids conventional serological tests and shows the reaction of sera with *T. gondii* antigens on a blotting membrane after they

are separated and transferred from a polyacrylamide gel. The resultant band patterns are compared to a standard molecular weight marker. A Western blot (WB) test can have varying reliability of its sensitivity and specificity depending on the type of sample used (Villard et al., 2003; Stroehle et al., 2005). WB is also complementary for the timely postnatal diagnosis of congenital toxoplasmosis (Robert-Gangneux et al., 1999), diagnosis of human patients (Gay et al., 2019) and characterization and evaluation of *T. gondii* proteins (Khanaliha et al; 2012; Ching et al., 2013; Ching et al., 2014; Appiah-Kwarteng et al., 2019; Liu et al., 2019).

3. Production of specific and standard antigens for serological diagnosis of toxoplasmosis

While *T. gondii* diagnosis based on serology are generally satisfactory, it has been challenged with producing specific and standard antigens that are commonly crudely produced through passages in mouse or by cell culture systems in commercial tests (Titilincu et al., 2009; Cóceres et al., 2010; Dai et al., 2012; Sudan et al., 2013; Sun et al., 2013). It has been shown that the different processes of producing and purifying native antigens may lead to contamination with non-parasitic materials (Holec-Gąsior, 2013). These processes also utilize live pathogens that require extra care because of biological hazards (Sonaimuthu et al., 2014) and are, therefore, difficult to standardize (Holec-Gąsior, 2013). With the limitations posed by the native antigens and the need to improve serodiagnostic tests, recombinant antigens have been considered as an alternative diagnostic marker to replace the native antigens (Pietkiewicz et al., 2004; Beghetto et al., 2006; Holec-Gąsior, 2013).

4. Toxoplasmosis in the Philippines

T. gondii infections have been documented in many different countries in Southeast Asia. In the Philippines, studies that reported toxoplasmosis include a few histopathological studies in rats and cats and several reports in pigs, rats, and cats mainly using serological methods (Salibay and Claveria, 2005; Advincula et al., 2010; Chua et al., 2014). Presently, reports on *T. gondii* infections in humans in the Philippines have been limited to Metro Manila, Mindoro, Cavite, Samar, and Leyte only (Cross et al., 1977; Kawashima et al., 2000; Salibay et al., 2008). In Cebu, the only report was in slaughter pigs in 1982 (Manuel, 1982), and none in humans and cats, so far.

5. General objectives of the present study

For several years, there have been several methods established for the diagnosis of toxoplasmosis, and many have produced satisfactory results. Nevertheless, to effect large-scale assessment of the presence of *T. gondii* infection, the development of specific, reliable, and fast approaches for its serodiagnosis remains a challenge. While several countries are aware of the disease and have already implemented routine screening for toxoplasmosis, some (including the Philippines) are still unaware of the current status of the infection due to lack of nationwide epidemiological studies. Thus, this study generally aimed to:

1) update the epidemiological status of the seroprevalence of *T. gondii* in humans, pigs, and cats in Cebu, Philippines;
2) assess the reliability of an ICT using TgGRA7 in the detection of *Toxoplasma* infections in humans; and,

3) evaluate the efficacy of an ICT using TgGRA7 in the detection of *Toxoplasma* infections in cats.

Chapter 1

Endemicity of *Toxoplasma* infection and its associated risk factors in Cebu, Philippines

1-1. Introduction

Toxoplasmosis is a public health problem worldwide. It is caused by *Toxoplasma gondii*, an obligate, intracellular, parasitic protozoan. It is zoonotic and is capable of infecting nearly all warm-blooded animals, including humans, but cats are the only known definitive host. *T. gondii* reproduces in the gut of felids. After reproduction, the oocysts are shed through the feces. These environmentally-resistant oocysts can then contaminate the soil, which may be ingested by an intermediate host, such as rodents, birds, or other warm-blooded animals (Torrey and Yolken, 2013). This parasite can cause infections in humans following the ingestion of raw meat infected with tissue cysts, food or drink contaminated with oocysts, or by direct assimilation from the environment (Montoya and Lisenfeld, 2004; Dubey and Jones, 2008). Vertical transmission from the infected mother to the fetus may also occur (Paquet and Yudin, 2013). *T. gondii* infections in humans are generally asymptomatic or manifested by flu-like symptoms and lymphadenopathy (Dubey, 1991). In young children, pregnant women, congenitally-infected fetuses and newborns, and immunocompromised people such as those with HIV/AIDS, those undergoing chemotherapy or those who have received an organ transplant, toxoplasmosis may be severe (Dubey and Jones, 2008; Weiss and Dubey, 2009). In most livestock animals such as poultry, pigs, and cattle, clinical toxoplasmosis is rare (Dubey

and Jones, 2008); however, just like in humans, it mostly affects the young and fetuses. Thus, it is considered one of the most common zoonotic infections causing abortions in both humans and female animals (Tenter et al., 2000; Hill and Dubey, 2002). Clinical signs rarely present themselves in infected cats, and if present, are caused by tissue inflammation and necrosis induced by the intracellular growth of tachyzoites (Dubey and Lappin, 2006). Like most human and animal cases, congenital infections in cats are more severe than in adults (Dubey, 1994; Dubey and Lappin, 2006).

The course of *T. gondii* infection may be affected by different conditions, such as the different sources and degrees of exposure to the parasite, and the immunological status of the individual (Dalimi and Abdoli, 2012). Cases also vary between countries and regions, being affected by other factors (Furtado and Smith, 2011) such as the climate, which affects the conditions for survival of the oocysts in the environment, and anthropogenic factors as reflected by the individual's hygiene and diet (Robert-Gangneux and Dardé, 2012). Other risk factors for acquiring human *T. gondii* infection, include age (Kawashima and Kawabata, 2000; Ertug et al., 2005; Alvarado-Esquivel et al., 2016), sex (Jones et al., 2009), close association with cats (Salibay et al., 2008; Chiang et al., 2012; Agmas et al., 2015), cleaning cat litter (Kapperud et al., 1996) and a lower educational level (Chiang et al., 2012; Agmas et al., 2015). In some areas, seroprevalence has been reported to vary among residents of rural, suburban and urban areas, with significantly higher seroprevalence in rural and suburban areas than in urban areas (Kawashima and Kawabata, 2000; Salibay et al., 2008). This variation may be attributed to the difference in socioeconomic status and hygienic practices, factors which are linked to the ingestion of oocysts from contaminated water, crops, animal products, and unpasteurized milk, likely contaminated from oocysts shed by cats or contaminated soil

(Kawashima and Kawabata, 2000; Ertug et al., 2005). Generally, contact with cats and consumption of raw or undercooked meat were the most common risk factors in acquiring toxoplasmosis.

For non-feline animals, contact with cats increases an animal's susceptibility to infection, which may be through direct contact or by indirect contact through a water source (Cenci-Goga et al., 2013). In pigs, the farming system affects the *Toxoplasma* seroprevalence (Dubey, 2009; Guo et al., 2015). Serodetection is significantly higher among extensively farmed pigs compared with intensively farmed pigs (Dubey and Jones, 2008) as a result of outdoor roaming, which increases contact with infected rodents, dead animals and oocyst-contaminated feed, water and surfaces (Guo et al., 2015). For cats, seroprevalence increases with age as a result of increased contact with infection sources, but younger cats can also become infected (Castillo-Morales et al., 2012). Poor body condition (Castillo-Morales et al., 2012; Tehrani-Sharif et al., 2015), access to the outdoors, the presence of dogs in the household, hunting, a history of being stray, and feeding on raw meat (Lopes et al., 2008; Opsteegh et al., 2012) have been identified as risk factors for infection in cats. Studies also reported that location (urban, suburban, and rural areas), diet, and domestication (Garcia et al., 2014) influence seropositivity in animals. Table food may contain tissue cysts thereby causing infection in cats (Lopes et al., 2008; Garcia et al., 2014).

The Sabin–Feldman *Toxoplasma* dye test is considered the gold standard for detecting anti-*Toxoplasma* antibodies in humans. As the parasite is not always detectable in patient samples, serological tests, such as an enzyme-linked immunosorbent assay (ELISA), a latex agglutination test (LAT), and an immunochromatographic test (ICT) are mostly used. For the detection of the organism in patient samples, PCR is widely used (Hill and Dubey, 2002).

Toxoplasmosis has been reported in many different countries in Southeast Asia. In the Philippines, studies that have documented toxoplasmosis have mainly used serological methods, with several reports in pigs, rats, cats and a few histopathological studies in rats and cats (Salibay and Claveria, 2005; Advincula et al., 2010; Chua et al., 2014). To date, three studies have documented *T. gondii* infections in humans in Metro Manila, Mindoro, Cavite, Samar, and Leyte (Cross et al., 1977; Kawashima et al., 2000; Salibay et al., 2008). In Cebu, the only report was in slaughter pigs in 1982, which documented a detection rate of 29.5% (Manuel, 1982). There are no studies yet on toxoplasmosis in humans and cats in this area. Thus, this study aimed to update the epidemiological status of the seroprevalence of *T. gondii* in humans, pigs, and cats in Cebu. It also sought to assess risk factors associated with the infection to increase the awareness of toxoplasmosis as a major public health threat in Cebu, Philippines.

1-2. Materials and methods

Sample population and sampling area

A total of 594 humans, 514 pigs, and 104 cats were sampled in Cebu, Philippines. For humans, adult participants (18 years old and above) from rural, suburban, and urban areas in Cebu were chosen by convenience sampling (Fig 1). The respondents must have been a resident of the area for at least six months to be included. Respondents were recruited from September 2016 to April 2018. The cat samples came from households, rescue shelters, and the streets of Cebu City and Liloan, Cebu, Philippines (Fig 2). The pig samples were obtained from Talisay City Slaughterhouse, Talisay City, Cebu, Philippines. The sampled pigs came

from different areas in the province (Talisay, Pinamungajan, Barili, San Fernando, Cebu City, Naga City, Carcar, Bantayan Island, Minglanilla, Carmen and Compostela) (Fig 3). The pigs had slaughter weights of 95–110 kg. Cat and pig serum samples were collected during April 2018. The respondents and animal profiles and risk factors were determined using survey questionnaires (S1–S3 Figs).

Profile parameters

Out of 924 respondents, most were women (60.9%) who were not pregnant (96.8%), single (63%), students (52.6%), and residing in an urban area (58%). The study population comprised predominantly of the younger age group (mean age = 32 years). This is representative of the general population in the Philippines, which has a large proportion of younger people. The majority of the respondents had no current (88.1%) or previous medical conditions (92.5%) and most consumed street foods (85%). The majority also did not have a pet cat (38.3%) or had no contact with cats (38.3%) (Table 1).

Only one cat out of 104 tested in this study showed poor body condition. The majority of the cats were male (54.8%), in rescue shelters (47.1%), not neutered or spayed (60.6%), and were fed a mixed table food diet (53.8%). Furthermore, the majority of cats had no litter trays (75%), were allowed outdoors (85.6%) and had contact with other animals (95.2%) (Table 2). By comparison, most of the pigs tested were male (55.8%) and were raised in extensive or backyard farms (55.6%) (Table 3).

Blood sampling and serum collection

Blood samples were aseptically collected by venipuncture of the cephalic vein for humans and the jugular vein for cats by a licensed phlebotomist and veterinarian, respectively. Blood from pigs was obtained in the bleeding process during slaughter. Serum samples were separated by centrifugation and stored at -20°C until further use. The profile of participants was obtained using a questionnaire that asked for relevant information, including some risk factors for toxoplasmosis.

Testing of sera using a latex agglutination test (LAT)

The sera were tested for antibodies against *T. gondii* using a commercial LAT kit (Toxocheck-MT; Eiken Chemical, Tokyo, Japan). The test was performed according to the manufacturer's recommendation. Briefly, 25 µL of latex solution was added into a round-bottom 96-well plate and 63 µL of LAT buffer into another round-bottom 96-well plate. The serum sample (1 µL) was added to the LAT buffer. After pipetting the serum and LAT buffer to mix, 25 µL of the serum-buffer mixture (1:64) was added to 25 µL of latex solution. The plate was sealed and allowed to stand at room temperature for 12 hr. Agglutination or visible clumping spreading throughout the well was interpreted as a positive reaction. A small, distinct circular precipitation in the center was indicative of a negative response. Positive and negative reference serum samples used in the assay were sera confirmed to be positive or negative using a commercially available ELISA kit (Platelia Toxo IgG/IgM; Bio-Rad, Hercules, CA, USA).

Data processing and analyses

Data from the questionnaire and LAT results were manually tabulated in a tally sheet and encoded to Microsoft Excel using appropriate coding to facilitate statistical analyses. Descriptive statistics were employed where applicable. The statistical significance of the test results and profiles were evaluated using a Chi-square and Mann–Whitney U test. *P*-values of less than 0.05 were considered significant. Odds ratio (OR) and confidence intervals (CI) were also computed.

Ethical clearance

The study was conducted according to the principles of the Helsinki declaration developed by the World Medical Association. Written informed consent (S4 Fig) was obtained from the respondents or their representatives after a careful explanation of the study. Written permission from cat owners and rescue shelter workers (S5 Fig) was also sought before blood collection. Ethical clearance of this study in humans was reviewed and approved by the Institutional Review Board of the University of the Visayas, Cebu (UV-IRB2017-91). Sampling in animals was conducted in accordance with the Animal Welfare Act of the Philippines (RA 8485) and the Bureau of Animal Industry Administrative Order 40, and the protocol was reviewed and approved by the Gullas College of Medicine Institutional Animal Care and Use Committee (Protocol no. 2017-01-01).

1-3. Results

Out of 924 participants, 244 (26.4%) were found to be seropositive (Table 1) from 21 different municipalities and cities in Cebu (Fig 1). Seropositivity was found to be greater among women (27%) than men (25.5%); however, sex was not found to be significantly associated with *T. gondii* seropositivity ($P = 0.611$). Students (28%) and respondents with non-health-related occupations (24.2%) were found to have higher seropositivity rates than health-related professionals (18.9%). Additionally, more seropositive participants had pet cats (35.2%) or were in frequent contact with these animals (45.4%). A higher proportion of seropositive individuals were suburban residents (35.1%) and consumed street foods (27.6%). Statistical analyses revealed no significant differences in seropositivity associated with sex or age. Furthermore, the respondents' occupation was not significantly associated with seropositivity ($P = 0.060$). However, living in a suburban area ($P = 0.002$), cat ownership ($P = 0.021$), frequent contact with cats ($P < 0.0001$) and consumption of street foods ($P = 0.044$) were all found to be significantly associated with seropositivity to *T. gondii* in humans (Table 1). These factors were associated with an increased risk of being seropositive for *T. gondii* in humans with odds ratios of 1.66 (95% CI 1.20–2.31), 1.48 (95% CI 1.07–2.07), 2.43 (95% CI 1.73–3.42), and 1.58 (95% CI 1.01–2.48), respectively. Of the pregnant women tested in this study, 4 out of 18 (16.7%) were seropositive for *T. gondii* antibodies.

For cats, the seropositive detection rate was 42.3% (44 of 104), with seropositive cats detected in Liloan and Cebu City (Fig 2). Seropositivity was higher among stray cats (7/8; 87.5%) than household cats (21/47; 44.7%) (Table 2). However, the small sample size for stray cats in this study should be taken into consideration. Although the difference was not statistically significant ($P = 0.400$), more seropositive female cats (46.7%) were detected than

male cats (38.6%). Greater seropositivity was observed in fertile cats (46%) than in neutered or spayed cats (36.6%). In this study, only one cat had poor body condition and this cat was negative for *T. gondii* antibodies. Body condition was found to have no significant association with seropositivity in cats ($P = 0.623$). The use of litter trays ($P = 0.002$) (Table 2) was found to be significantly associated with seropositivity in cats and increased the odds of cats becoming infected with *T. gondii* (OR 4.5, 95% CI: 1.73–11.71).

Serological testing revealed that 13.4% (69 of 514) of pigs were seropositive. Seropositive animals came from 10 municipalities/cities in Cebu (Fig 3). Most of the pigs that tested positive were female (14.1%) and were raised through non-intensive or backyard farming (14.3%) (Table 3). However, sex ($P = 0.691$) and the rearing system ($P = 0.497$) were not significantly associated with seropositivity in pigs.

1-4. Discussion

The seroprevalence of *T. gondii* in humans in this study (26.4%) was slightly lower than the previously reported detection level (27.1%) in the Philippines using the same detection method (Salibay et al., 2008). Another report in the country showed a lower detection rate using ELISA on samples from Metro Manila (11.1%), but higher rates for Mindoro (61.2%) and Leyte (30.1%) (Kawashima et al., 2000). The higher seropositivity rates among women in this study (27.0%) were similar to the findings of Salibay et al. (2008) that reported 32% seropositivity. However, sex was not found to be significantly associated with seropositivity in previous or current studies in this country. The 16.7% detection rate among pregnant women in this study was comparable to that of previous studies in pregnant women in Singapore, which showed a prevalence of 17.2% (Wong et al., 2000), and in Vietnam with a seroprevalence of

11.2% (Buchy et al., 2003), using immunofluorescent antibody test (IFAT) and ELISA, respectively. In Thailand, a slightly higher seroprevalence of 22% was reported in 2014 using ELISA (Andiappan et al., 2014). Using the same method, 7.7% seropositivity was recently published in Taiwan (Hung et al., 2015), which is lower than that detected in the present study. However, the number of pregnant women tested was low, which is also a limitation of the current study. All of the above-mentioned reports did not find any significant association between seropositivity and risk factors, with the exception of the study by Andiappan et al. (2014) that reported age, occupation (as laborers) and drinking water source as significant risk factors for *Toxoplasma* seropositivity in pregnant women in Thailand ($P < 0.05$).

My study revealed no significant differences in the seropositivity rates relating to the age or sex of respondents, which was similar to previous findings in some areas of the Philippines (Auer et al., 1995; Salibay et al., 2008). Kawashima et al. (2000), however, noted that infection rates in the Philippines increased with age. This implied that *T. gondii* may infect any sex at any age, but the frequency of seropositivity is higher among older individuals, most likely because they have been exposed to risk factors longer than the younger individuals. Previous studies in the Philippines reported significantly higher seroprevalence in rural and suburban areas than in urban areas (Kawashima and Kawabata, 2000; Salibay et al., 2008), which was consistent with the findings of my study. This variance among locations may be attributed to the socioeconomic status of the participants, which may affect hygiene practices and the likelihood of ingestion of oocysts from water, crops, animal products, and unpasteurized milk contaminated from oocysts shed by cats and contaminated soil (Kawashima and Kawabata, 2000; Ertug et al., 2005). A study in the Philippines also indicated higher seroprevalence among individuals with apparent association with cats (Salibay et al., 2008).

Worldwide, the close association with, or ownership of cats have been identified as vital risk factors for human infection. Being the definitive host of *T. gondii,* cats have a primary role in the transmission of the parasite, and frequent contact with cats generally increases the risk of infection due to contact with oocysts in cat feces (Chiang et al., 2012; Agmas et al., 2015).

The reported serodetection of *Toxoplasma* in pregnant women in this study may have a potential detrimental effect on the fetus due to the risk of congenital infection. Congenital infection is a crucial part of the disease burden in human *Toxoplasma* infections (Robert-Gangneux and Dardé, 2012). This study is the first to serologically detect *T. gondii* infection in pregnant women in the Philippines. Unlike in other countries, screening is not routinely performed in this country. However, the widespread serodetection of *Toxoplasma* in this study implies the necessity for an increased sampling area and size to clarify the epidemiological status of this pathogen in the Philippines.

The serodetection rate observed in cats in this study is similar to that previously reported in the northern Philippines (46.67%) (Advincula et al., 2010), but is lower than the reported seroprevalence of 72.3% in Vietnam (Hosono et al., 2009). A recent study in Japan, however, only documented *T. gondii* infection rate of 6.7% in cats (Oi et al., 2015). Advincula et al. (2010) found that male cats were more susceptible to infection; however, other reports, including this study, concluded that sex is not a risk factor for toxoplasmosis in cats (Smielewska-Loś and Pacoń, 2002; Gauss et al., 2003). The greater seropositivity observed in fertile cats in this study may be due to the behavioral habits of fertile cats to roam in search of a mate. Poor body condition was also reported to affect seropositivity in cats (Castillo-Morales et al., 2012; Tehrani-Sharif et al., 2015). However, in this study, body condition showed no signification association with seropositivity in cats. Furthermore, the current study showed the

increased likelihood of cats to become infected with *T. gondii* through the use of litter trays. According to the Center for Disease Control, exposure to cat litter trays may facilitate contact with infective *T. gondii* oocysts. Generally, location (urban, suburban, and rural areas), diet and domestication influence seropositivity in animals (Lopes et al., 2008; Opsteegh et al., 2012). Seropositivity for infection was significantly higher in suburban than in urban areas for cats in Japan (Oi et al., 2015) and was also higher among strays than domesticated cats and dogs in Korea and in other parts of the Philippines (Lee et al., 2010; Garcia et al., 2014; Guy and Penuliar, 2016), which is consistent with the results of this study.

In 1982, the first and only report of toxoplasmosis in Cebu, Philippines, documented a 29.50% (59 of 200) seropositivity rate among slaughter pigs (Manuel, 1982). My study investigated the current infection status in swine in Cebu. The seroprevalence rate in pigs was found to be lower than the initial report by Manuel (1982), as well as the prevalence (27.2%) reported in Vietnam (Huong and Dubey, 2007). However, it was similar to a previous report in Metro Manila (13.6%) (Manuel and Tubongbanua, 1977) and reports from other neighboring countries, such as Taiwan (10.1%) (Tsai et al., 2007) and China (10.4%) (Lin et al., 1990). Lower seroprevalence was reported in Indonesia and Japan at 2.3% and 5.2%, respectively (Matsuo et al; 2014; Tuda et al., 2017). The differences in seroprevalence reported by these studies may be attributed to the various serodiagnostic techniques and cut-off values employed (Dubey, 2009). Although the rearing system was not significantly associated with seropositivity in pigs in this study ($P = 0.500$), the method by which pigs are farmed affects seroprevalence by potentially predisposing the animals to infection (Dubey, 2009; Guo et al., 2015). The substantially higher serodetection rate among pigs farmed by extensive (non-intensive/backyard) production methods compared with intensively farmed pigs is due to the

access of these animals to the outdoors, which increases contact with infected rodents and dead animals and oocyst-contaminated feed, water and surroundings, as reported in the USA and Japan (Dubey and Beattie, 1988; Dubey and Jones, 2008; Guo et al., 2015). Access of cats to farm animals such as pigs has also been identified as a critical risk factor for the transmission of *T. gondii* infection (Herrero et al., 2016). The serological detection of *T. gondii* in pigs in Cebu three decades after its first detection revealed the constant presence of *T. gondii* in the area. In general, seropositivity gauges the presence of *T. gondii* in tissues (Dubey et al., 2005; Dubey and Jones, 2008). The findings of my study suggest the endemicity of *T. gondii* infection in pigs reared in Cebu. This presents a public health hazard, as *T. gondii* cysts in pork may be infectious if the meat is undercooked.

The prevalence rates obtained in this study were dependent on the detection method used. The LAT detects total antibody and does not distinguish between recent and past exposures. The kit used in this study had been used in several serological surveys with various animal species including humans because of its ease of use, low cost, and high sensitivity, and specificity (Salibay et al., 2008; Matsuo et al; 2014). Because the performance of assays can differ, relative seroprevalence rates may also vary depending on the test used. Thus, statistical correlations on risk factors may also give varying results depending on the assay used in a study.

While several other countries are aware of the disease and have already implemented routine screening for toxoplasmosis, most communities in the Philippines remain unaware of this disease. It is estimated that only 11% of pregnant women in the Philippines are aware of the disease (Andiappan et al., 2014). The positive serodetection rates among pregnant women in this study suggest the possible presence of infection in other parts of the Philippines. While

serological evidence of exposure of pigs, humans, and cats to *T. gondii* was presented, the present study could not account for the status of the disease across the entire country because of sampling limitations. Future studies with an increased sampling area and size could help clarify the epidemiological status of *T. gondii* in this country and determine whether screening among the general population is essential. As my study detected seropositive cats in areas with confirmed cases of seropositive humans, there is a need to conduct further studies in stray cats that are commonly found in areas selling street foods, with transmission possibly resulting from the consumption of infected rats or meat. Moreover, seropositivity was also detected among pigs in the country, which places the predominantly meat-eating Filipino people at higher risk of consumption of potentially undercooked infected meat. Food preparation, hygienic and sanitary practices may also lead to the contamination of food from uncooked contaminated meat and other contaminated food.

1-5. Summary

This study reports the first serological detection of *T. gondii* in humans and cats in Cebu, Philippines, and the most recent serological status update in pigs since 1982 in the area. To the best of my knowledge, this is also the first report documenting serodetection of *T. gondii* in pregnant women in the country. The confirmed seropositivity of *T. gondii* in Cebu, Philippines, in the present study implies the endemicity of this disease in the area and highlights the need for increased public awareness in the country. Routine testing may be recommended for vulnerable populations, including pregnant women, immunocompromised individuals, and those exposed to cats. An educational campaign about toxoplasmosis is needed to increase awareness among the public.

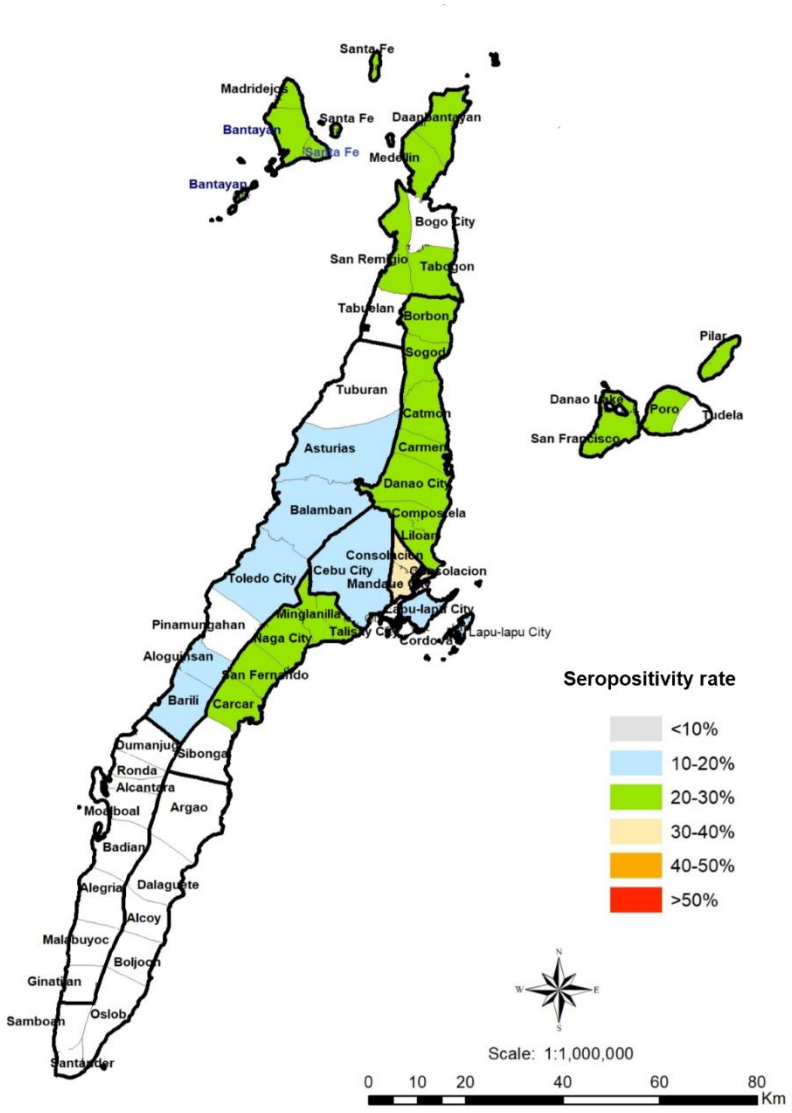


Fig 1. Geographical distribution of *T. gondii* infections of humans in Cebu, Philippines. Colored areas are those with *T. gondii*-seropositive respondents. Urban: Cebu City, Mandaue City, Lapu-Lapu City; Suburban: Minglanilla, Talisay, Naga, San Fernando, Carcar, Consolacion, Liloan, Danao, Compostela; Rural: Barili, Argao, Alegria, Dumanjug, Daanbantayan, Carmen, Sogod, Catmon, San Remigio, Borbon, Bantayan Island, Bogo, Madridejos, Poro, San Francisco, Danao Lake, Pilar. QGIS software, a free and open source geographic information system, was used to create the map (https://www.qgis.org/). PhilGIS, an open and free source for GIS data for the Philippines, was used to integrate the municipal boundaries or base layer (http://philgis.org/province-page/cebu).

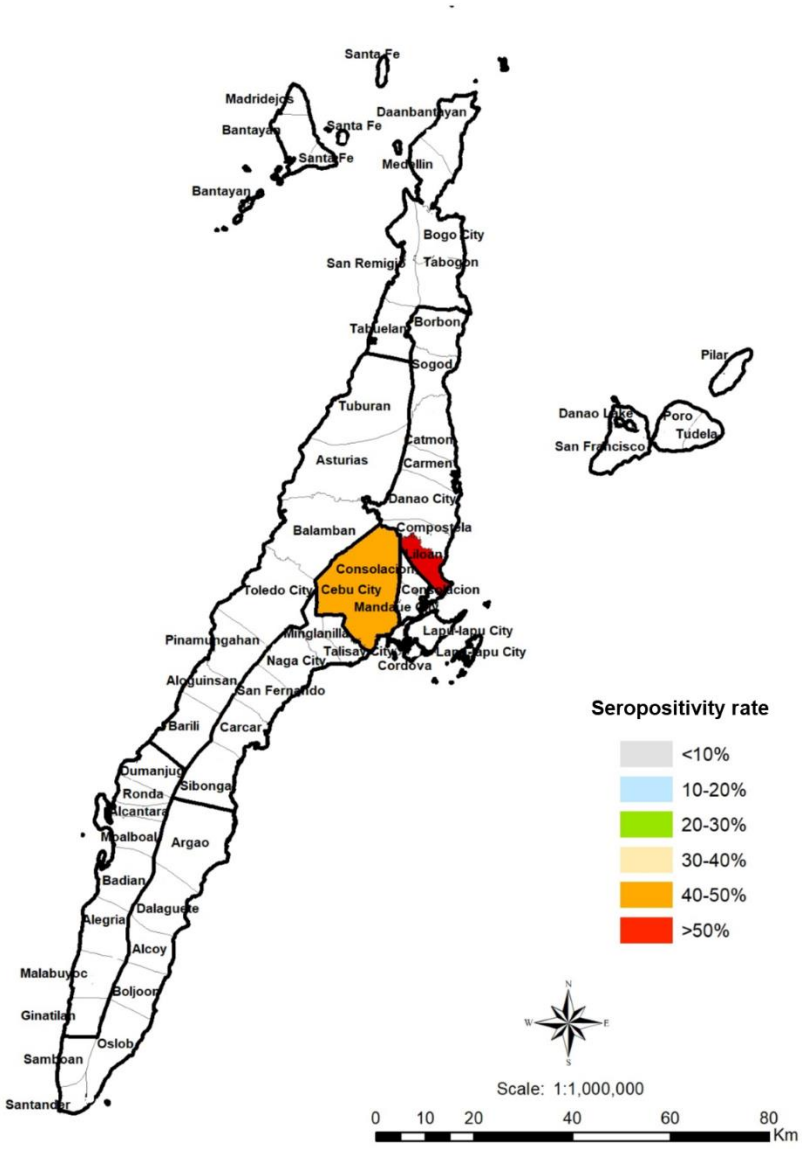


Fig 2. Geographical distribution of *T. gondii* infections in cats in Cebu, Philippines. Colored areas are those with *T. gondii*-seropositive cats. QGIS software was used to create the map (https://www.qgis.org/). PhilGIS was used to integrate the municipal boundaries or base layer (http://philgis.org/province-page/cebu).

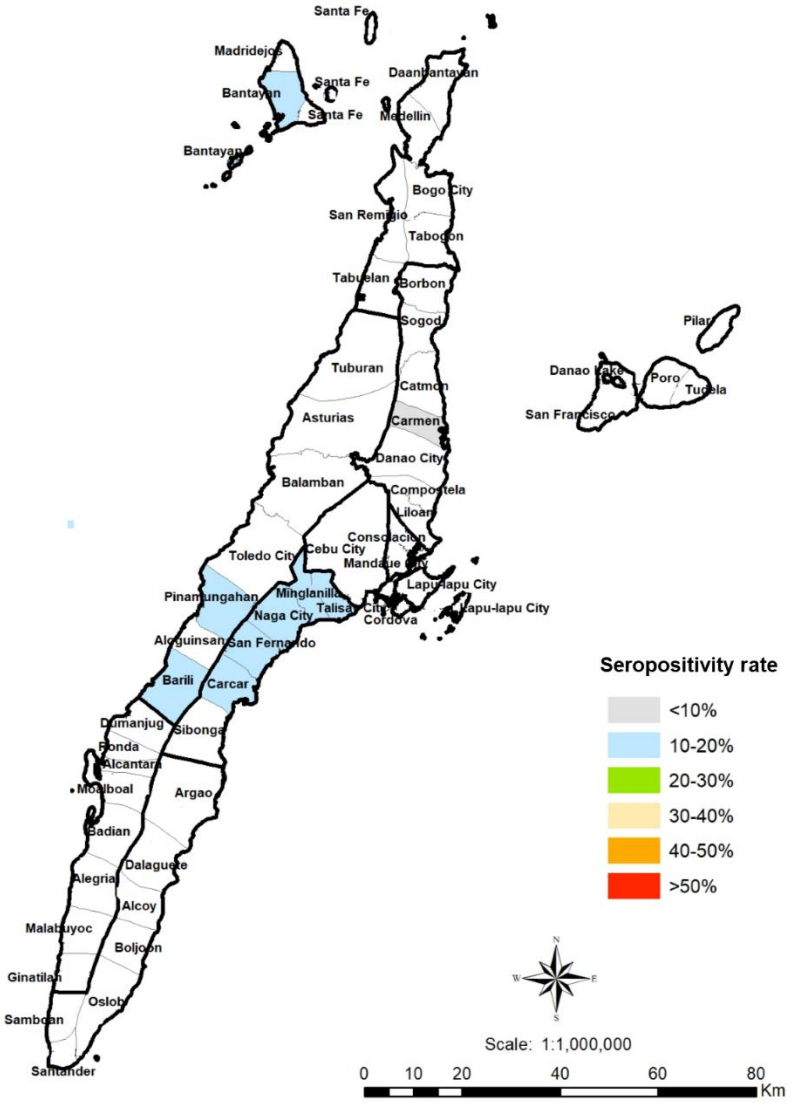


Fig 3. Geographical distribution of *T. gondii* infections in slaughter pigs in Cebu, Philippines. Colored areas are those with *T. gondii*-seropositive pigs. QGIS software was used to create the map (https://www.qgis.org/). PhilGIS was used to integrate the municipal boundaries or base layer (http://philgis.org/province-page/cebu).

ID No. _____

Seroprevalence of *Toxoplasma gondii* in humans in Cebu

Main Researchers/Project Leaders: **Rochelle Haidee D. Ybañez[1] and Adrian P. Ybañez[1,2]**
[1]Biology and Environmental Studies Program, Sciences Cluster, University of the Philippines Cebu, Gorordo Avenue, Lahug, Cebu City
[2]Gullas College of Medicine, University of the Visayas, Gov. M. Cuenco Ave, Cebu City

Profile (Please provide the necessary information or mark √ to applicable answers)

Name: _____

Address: _____

Age: _____ years Sex: ◻ Male ◻ Female Civil Status: ◻ single ◻ married ◻ widowed

Highest Educational Attainment: ◻ Elementary Level ◻ Elementary Graduate
◻ High School level ◻ High School Graduate
◻ College Level ◻ College Graduate
◻ Others (please specify) _____

Profession/Work: _____

Present medical conditions: ◻ None ◻ Others (Please indicate) _____

Previous medical conditions/procedures: ◻ None ◻ Others (Please indicate) _____

Do you own a cat? ◻ No ◻ Yes (please indicate number) _____

If yes, how long have you been living with cats? _____ years

Do you have constant contact with cats? ◻ No ◻ Yes (please estimate or describe frequency)

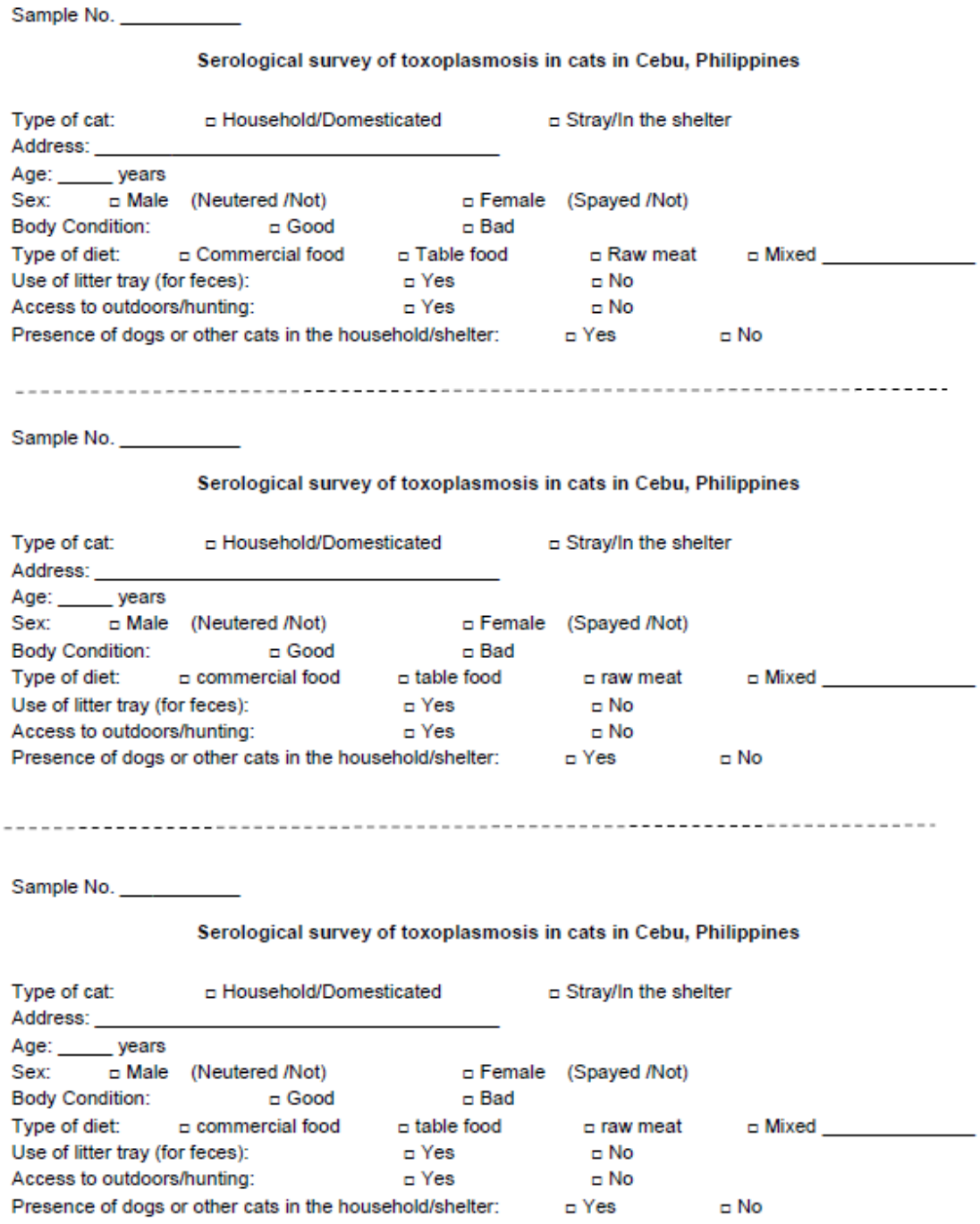
_____

Do you own other animals aside from cats? ◻ No ◻ Yes (please indicate) _____

Do you eat street foods? ◻ No ◻ Yes (indicate where) _____

Are you currently pregnant? ◻ No ◻ Yes

S1 Fig. Questionnaire used during sample collection with respondents. The questionnaire was used to obtain the profile of the respondents which was used during the analysis.



Sample No. _____

Serological survey of toxoplasmosis in cats in Cebu, Philippines

Type of cat: ◻ Household/Domesticated ◻ Stray/In the shelter
Address: _____
Age: _____ years
Sex: ◻ Male (Neutered /Not) ◻ Female (Spayed /Not)
Body Condition: ◻ Good ◻ Bad
Type of diet: ◻ Commercial food ◻ Table food ◻ Raw meat ◻ Mixed _____
Use of litter tray (for feces): ◻ Yes ◻ No
Access to outdoors/hunting: ◻ Yes ◻ No
Presence of dogs or other cats in the household/shelter: ◻ Yes ◻ No

---

Sample No. _____

Serological survey of toxoplasmosis in cats in Cebu, Philippines

Type of cat: ◻ Household/Domesticated ◻ Stray/In the shelter
Address: _____
Age: _____ years
Sex: ◻ Male (Neutered /Not) ◻ Female (Spayed /Not)
Body Condition: ◻ Good ◻ Bad
Type of diet: ◻ commercial food ◻ table food ◻ raw meat ◻ Mixed _____
Use of litter tray (for feces): ◻ Yes ◻ No
Access to outdoors/hunting: ◻ Yes ◻ No
Presence of dogs or other cats in the household/shelter: ◻ Yes ◻ No

---

Sample No. _____

Serological survey of toxoplasmosis in cats in Cebu, Philippines

Type of cat: ◻ Household/Domesticated ◻ Stray/In the shelter
Address: _____
Age: _____ years
Sex: ◻ Male (Neutered /Not) ◻ Female (Spayed /Not)
Body Condition: ◻ Good ◻ Bad
Type of diet: ◻ commercial food ◻ table food ◻ raw meat ◻ Mixed _____
Use of litter tray (for feces): ◻ Yes ◻ No
Access to outdoors/hunting: ◻ Yes ◻ No
Presence of dogs or other cats in the household/shelter: ◻ Yes ◻ No

S2 Fig. Questionnaire used during sample collection in cats. The questionnaire was used to obtain the profile of the cats which was used during the analysis.

Sample No. ___________

Serological survey of toxoplasmosis in slaughter pigs in Cebu, Philippines

Location of Source of Pigs: ______________________________
Rearing system: □ Intensive (Closed environment) If intensive: □ Conventional □ Organic
□ Non-intensive (Open/Backyard)
Age: _____ months (estimate if exact age is unknown)
Sex: □ Male □ Female

Sample No. ___________

Serological survey of toxoplasmosis in slaughter pigs in Cebu, Philippines

Location of Source of Pigs: ______________________________
Rearing system: □ Intensive (Closed environment) If intensive: □ Conventional □ Organic
□ Non-intensive (Open/Backyard)
Age: _____ months (estimate if exact age is unknown)
Sex: □ Male □ Female

Sample No. ___________

Serological survey of toxoplasmosis in slaughter pigs in Cebu, Philippines

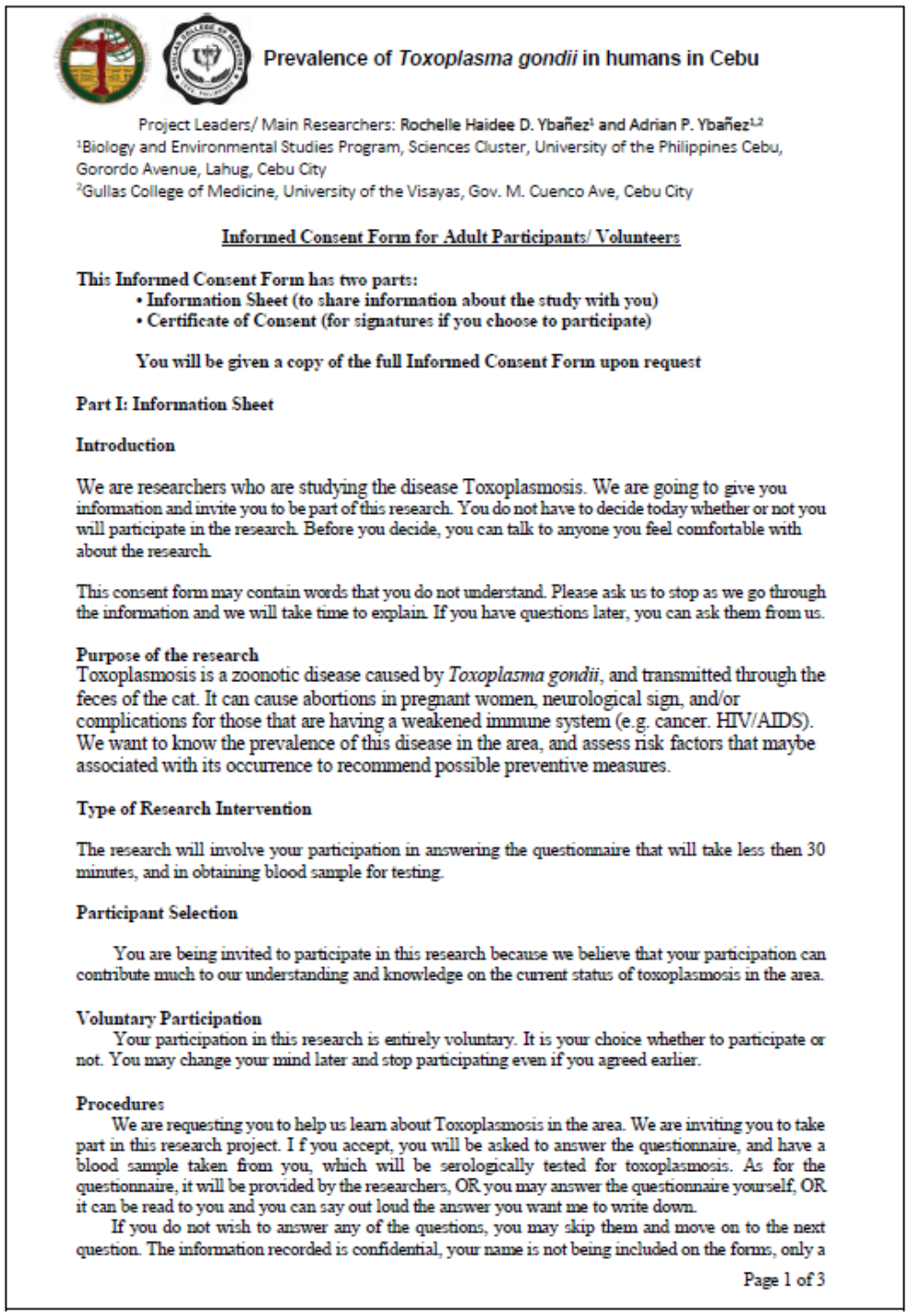
Location of Source of Pigs: ______________________________
Rearing system: □ Intensive (Closed environment) If intensive: □ Conventional □ Organic
□ Non-intensive (Open/Backyard)
Age: _____ months (estimate if exact age is unknown)
Sex: □ Male □ Female

Sample No. ___________

Serological survey of toxoplasmosis in slaughter pigs in Cebu, Philippines

Location of Source of Pigs: ______________________________
Rearing system: □ Intensive (Closed environment) If intensive: □ Conventional □ Organic
□ Non-intensive (Open/Backyard)
Age: _____ months (estimate if exact age is unknown)
Sex: □ Male □ Female

S3 Fig. Questionnaire used during sample collection in slaughter pigs. The questionnaire was used to obtain the profile of the pigs which was used during the analysis.



Prevalence of *Toxoplasma gondii* in humans in Cebu

Project Leaders/ Main Researchers: Rochelle Haidee D. Ybañez[1] and Adrian P. Ybañez[1,2]
[1]Biology and Environmental Studies Program, Sciences Cluster, University of the Philippines Cebu, Gorordo Avenue, Lahug, Cebu City
[2]Gullas College of Medicine, University of the Visayas, Gov. M. Cuenco Ave, Cebu City

Informed Consent Form for Adult Participants/ Volunteers

This Informed Consent Form has two parts:
- **Information Sheet (to share information about the study with you)**
- **Certificate of Consent (for signatures if you choose to participate)**

You will be given a copy of the full Informed Consent Form upon request

Part I: Information Sheet

Introduction

We are researchers who are studying the disease Toxoplasmosis. We are going to give you information and invite you to be part of this research. You do not have to decide today whether or not you will participate in the research. Before you decide, you can talk to anyone you feel comfortable with about the research.

This consent form may contain words that you do not understand. Please ask us to stop as we go through the information and we will take time to explain. If you have questions later, you can ask them from us.

Purpose of the research
Toxoplasmosis is a zoonotic disease caused by *Toxoplasma gondii*, and transmitted through the feces of the cat. It can cause abortions in pregnant women, neurological sign, and/or complications for those that are having a weakened immune system (e.g. cancer. HIV/AIDS). We want to know the prevalence of this disease in the area, and assess risk factors that maybe associated with its occurrence to recommend possible preventive measures.

Type of Research Intervention

The research will involve your participation in answering the questionnaire that will take less then 30 minutes, and in obtaining blood sample for testing.

Participant Selection

You are being invited to participate in this research because we believe that your participation can contribute much to our understanding and knowledge on the current status of toxoplasmosis in the area.

Voluntary Participation
Your participation in this research is entirely voluntary. It is your choice whether to participate or not. You may change your mind later and stop participating even if you agreed earlier.

Procedures
We are requesting you to help us learn about Toxoplasmosis in the area. We are inviting you to take part in this research project. I f you accept, you will be asked to answer the questionnaire, and have a blood sample taken from you, which will be serologically tested for toxoplasmosis. As for the questionnaire, it will be provided by the researchers, OR you may answer the questionnaire yourself, OR it can be read to you and you can say out loud the answer you want me to write down.
If you do not wish to answer any of the questions, you may skip them and move on to the next question. The information recorded is confidential, your name is not being included on the forms, only a

Page 1 of 3

S4 Fig. Written consent form given to the respondents. A signed informed consent was obtained from the respondents after careful explanation of the study.

number will identify you, and no one else except the researchers will have access to your survey.

Duration

Questionnaire/interview will be short (around 30 minutes), while blood sampling will take around 3 minutes. The results of the serological testing will be provided to you in not more than 2 weeks after the blood sample is collected.

Risks

There is no major potential risks from the research, other than the possible information which you may deem personal. If you feel uncomfortable, you do not have to answer any question or take part in the discussion/interview/survey if you don't wish to do so, and that is also fine. You do not have to give us any reason for not responding to any question, or for refusing to take part in the interview.

Benefits

Other than knowing your status if you are positive with toxoplasmosis or not, there will be no direct benefit to you, but your participation is likely to help us find out more about the status of toxoplasmosis in the area and on how to prevent it in the community.

Reimbursements

You will not be provided any incentive to take part in the research. However, we will share with you your test results. The testing approximately costs PHP 1,500 to 2,500.

Confidentiality

The research may draw attention and if you participate, you may be asked questions by other people in the area. We will not be sharing information about you to anyone outside of the research team. The information that we collect from this research project will be kept private. Any information about you will have a number on it instead of your name. Only the researchers will know what your number is and we will lock that information up with a lock and key. It will not be shared with or given to anyone except the research sponsors.

Sharing the Results

Nothing that you tell us today will be shared with anybody outside the research team, and nothing will be attributed to you by name. The knowledge that we get from this research will be shared with you and your community before it is made widely available to the public. Each participant will receive a summary of the results. We will publish the results so that other interested people may learn from the research.

Right to Refuse or Withdraw

You do not have to take part in this research if you do not wish to do so, and choosing to participate will not affect your job or job-related evaluations in any way. You may stop participating in the interview/questionnaire at any time that you wish. We will give you an opportunity at the end of the interview/discussion to review your remarks, and you can ask to modify or remove portions of those, if you do not agree with our notes or if we did not understand you correctly.

Who to Contact

If you have any questions, you can ask them now or later. If you wish to ask questions later, you may contact any of the following: Adrian P. Ybanez, dr.adrianpybanez@gmail.com / 09228762439; Rochelle Haidee D. Ybanez, rdybanez1@up.edu.ph (09258237423).

This proposal has been reviewed and approved by UV-IRB, which is a committee whose task it is to make sure that research participants are protected from harm. If you wish to find about more about the IRB, contact Marites G. Arcilla, RN, MAN, DPE, DMc of the University of the Visayas. It has also been reviewed by CHED.

Part II: Certificate of Consent

I have been invited to participate in research about Toxoplasmosis. I have read the foregoing information about the research, or it has been read to me. I have had the opportunity to ask questions about it and any

questions I have been asked have been answered to my satisfaction. I consent voluntarily to be a participant in this study

Print Name of Participant (optional) _____
Signature of Participant _____
Date _____
Day/month/year

If illiterate [1]

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print name of witness _____ Thumb print of participant

Signature of witness _____

Date _____
Day/month/year

Statement by the researcher/person taking consent

I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the participant understands that the following will be done:
1. answering of the questionnaire
2. blood collection for testing with Toxoplasmosis3.

I confirm that the participant was given an opportunity to ask questions about the study, and all the questions asked by the participant have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

A copy of this ICF can be provided to the participant upon request.

Rochelle Haidee D. Ybañez/ Adrian P. Ybañez
Researchers

Date _____
Day/month/year

[1] A literate witness must sign (if possible, this person should be selected by the participant and should have no connection to the research team). Participants who are illiterate should include their thumb print as well.

Page 3 of 3

February 6, 2018

MR. DAN VETTER
Vice President
Mayari Animal Rescue Organization, Inc.

Dear Mr. Vetter,

Good day! I am a friend of Ms. Deena Kelly. I am currently pursuing my doctorate degree in Obihiro University of Agriculture and Veterinary Medicine in Japan. My research is on the development of serodiagnostic antigens of *Toxoplasma gondii* for its epidemiological study in the Philippines. Just to give you a brief background, *Toxoplasma gondii* is a microscopic protozoan parasite that is ubiquitous in nature. It causes the disease called Toxoplasmosis, which is considered one of the most common parasitic diseases around the world.

It is usually asymptomatic to healthy individuals or they may only develop flu-like symptoms because the immune system keeps the parasite from causing any illness. However, the disease is more problematic for pregnant women (causes abortion and/or abnormality in the babies such as hydrocephaly, brain or eye damage) and people who have weakened immune systems (causes brain disease, brain lesions, pneumonitis or retinochoroiditis). The parasites remain in the body as tissue cysts (bradyzoites) and reactivate, if the person becomes immunosuppressed by other diseases or by immunosuppressive drugs. Cats are the primary host of the parasite and humans and other warm blooded animals can be intermediate hosts.

Humans get toxoplasmosis through:

- consumption of undercooked, infected meat (especially pork, lamb, and chevon)
- consumption of fruits and vegetables or ingesting water, soil (i.e., putting dirty fingers in your mouth) or anything else that has been contaminated with cat feces
- mother-to-child transmission
    - A pregnant woman might not have any symptoms, but the unborn child might suffer and develop the disease.
- blood transfusion or organ transplantation (very rare)

In other countries, routine testing has already been conducted especially among pregnant women. However, not a lot of people know about toxoplasmosis in the Philippines. We have already conducted preliminary serological screening among veterinary personnel and pet owners in Cebu and other parts of the country. And there were individuals that tested positive of antibodies against the parasite. Those pet owners that tested positive had cats as pets.

In this regard, I would like to request for your kind approval to allow us to extract blood samples from the cats in the shelter so we could test them for toxoplasmosis. We have a team of veterinarians who can help facilitate the blood extraction for our cats and we can tap a phlebotomist to do the blood extraction for you and your personnel and volunteers should you decide to have your blood tested as well.

Your kind support and participation will really be of great help for the fulfillment of this research endeavor.

Thank you very much.

Respectfully yours,

ROCHELLE HAIDEE D. YBAÑEZ

S5 Fig. Written permission sent to rescue shelter owners. Letter was sent to cat owners and rescue shelter owners to seek approval prior to blood collection.

Table 1. Seroprevalence of toxoplasmosis in humans in Cebu, Philippines (N=924).

Profile	Positive (N=244)	Prevalence %	OR	95% CI	*P*-value
Sex					0.611
Female (N=563)	152	27.0	1.00		
Male (N=361)	92	25.5	1.08	0.80-1.46	
Age					0.472
18-30 years (N=556)	149	26.8	1.00		
31-40 years (N=155)	34	21.9	0.77	0.50-1.17	
41-50 years (N=105)	27	25.7	0.95	0.59-1.52	
51-60 years (N=52)	17	32.7	1.33	0.72-2.44	
61-70 years (N=41)	11	26.8	1.00	0.49-2.05	
71 years up (N=15)	6	40.0	1.82	0.64-5.20	
Civil Status					0.971
Single (N=582)	153	26.3	1.00		
Married (N=321)	85	26.5	1.01	0.74-1.38	
Widowed (N=21)	6	28.6	1.12	0.43-2.94	
Area					0.004*
Urban (N=536)	131	24.5	1.00		
Rural (N=148)	27	18.6	0.70	0.44-1.12	
Sub-urban (N=240)	86	35.1	1.66	1.20-2.31	
Occupation					0.060
Health related (N=169)	32	18.9	1.00		

Non-health related (N=231)	56	24.2	1.37	0.84-2.18	
Student (N=486)	136	28.0	1.66	1.08-2.56	
Preferred not to say (N=38)	20	52.6	4.76	2.26-10.01	
Present medical condition					0.109
No (N=814)	208	25.6	1.00		
Yes (N=110)	36	32.7	1.42	0.92-2.18	
Previous medical condition					0.829
No (N=855)	225	26.3	1.00		
Yes (N=69)	19	27.5	1.06	0.61-1.84	
Has pet cats					0.020*
No (N=354)	95	26.8	1.00		
Yes (N=301)	106	35.2	1.48	1.07-2.07	
Preferred not to say (N=269)	43	16.0	0.52	0.35-0.78	
Frequency of contact with cats					<0.0001*
None (N=412)	105	25.5	1.00		
Less frequent (N=278)	35	12.6	0.42	0.28-0.64	
Frequent (N=229)	104	45.4	2.43	1.73-3.42	
Prefer not to say (N=5)	0	0.0	0.27	0.01-4.48	
Consumption of street foods					0.043*
No (N=139)	27	19.4	1.00		
Yes (N=785)	217	27.6	1.58	1.01-2.48	
Pregnant Women					0.316
No (N=545)	149	27.3	1.00		

Yes (N=18)	3	16.7	0.53	0.15-1.86	0.323

Odds ratio (OR) was calculated by comparison with the first of each factor.

*Statistically significant with *T. gondii* seropositivity (*P*-value < 0.05)

Table 2. Serological survey of toxoplasmosis in cats in Cebu, Philippines (N=104).

Profile	Positive (N=44)	Prevalence %	OR	95% CI	*P*-value
Sex					
Female (N=47)	22	46.8	1.00		
Male (N=57)	22	38.6	1.40	0.64-3.06	0.400
Type					
Household or domesticated (N=47)	21	44.7	1.00		
Rescued or in shelter (N=49)	16	32.7	1.67	0.73-3.82	0.230
Stray (N=8)	7	87.5	0.12	0.01-1.01	0.051
Neutered/Spayed					
No (N=63)	29	46.0	1.00		
Yes (N=41)	15	36.6	0.68	0.30-1.51	0.340
Body condition					
Good (N=103)	44	42.7	1.00		
Bad (N=1)	0	0.0	0.45	0.02-11.20	0.623
Diet					
Commercial food (N=25)	13	52.0	1.00		
Table food (N=23)	9	39.1	1.69	0.53-5.31	0.373
Raw meat (N=0)	0	0.0			
Mixed (N=56)	22	39.3	1.67	0.65-4.33	0.288
Use of litter trays					
No (N=78)	26	33.3	1.00		

Yes (N=26)	18	69.2	4.50	1.73-11.71	0.002*
Access to outdoors / hunting					
No (N=9)	6	66.7	1.00		
Yes (N=95)	38	40.0	0.33	0.08-1.41	0.136
Contact with other animals					
No (N=4)	4	100.0	1.00		
Yes (N=100)	40	40.0	0.07	0.00-1.42	0.084

Odds ratio (OR) was calculated by comparison with the first of each factor.

*Statistically significant with *T. gondii* seropositivity (*P*-value < 0.05)

Table 3. Seroprevalence of toxoplasmosis in slaughter pigs in Cebu, Philippines (N=514).

Profile	Positive (N=69)	Prevalence %	OR	95% CI	*P*-value
Sex					
Female (N=227)	32	14.1	1.00		
Male (N=287)	37	12.9	0.91	0.54-1.50	0.69
Rearing system					
Intensive / Concrete (N=228)	28	12.3	1.00		
Non-intensive / Backyard (N=286)	41	14.3	1.20	0.71-2.00	0.50

Odds ratio (OR) was calculated by comparison with the first of each factor.

Chapter 2

Serological detection of *T. gondii* infection in humans using an immunochromatographic assay based on dense granule protein 7

2-1. Introduction

Toxoplasmosis, one of the world's most common parasitic diseases, is caused by *Toxoplasma gondii*, an obligate, intracellular, zoonotic parasite. This ubiquitous Apicomplexan infects humans and warm-blooded animals. Cats are the known definitive hosts of *T. gondii,* where it reproduces in the gut, and the environmentally-resistant oocysts are shed through the feces (Dubey and Beattie, 1988). Humans generally become infected after consumption of tissue cysts from raw or undercooked meat, oocyst-contaminated food or drinks, or by direct contact from the environment (Dubey and Jones, 2008; Torrey and Yolken, 2013). *T. gondii* infections in healthy humans are generally asymptomatic or may manifest only flu-like symptoms (Dubey, 1991). However, toxoplasmosis may have more severe effects in pregnant women, congenitally-infected fetuses and newborns, young children, and immunocompromised peoples such as HIV/AIDS patients, have undergone organ transplantation, or receiving chemotherapy (Dubey and Jones, 2008; Weiss and Dubey, 2009).

The detection of *T. gondii* is crucial not only for diagnosis and treatment but also for epidemiology and prevention. Commonly utilized diagnostic methods of *Toxoplasma* infection are based on the detection of specific antibodies, such as latex agglutination test (LAT), enzyme-linked immunosorbent assay (ELISA), and immunochromatographic test (ICT) (Hill

and Dubey, 2002). The LAT is a useful tool for serodiagnosis of *T. gondii* infection, but reports on false-positive reactions have been documented (Payne et al., 1984; Holliman et al., 1989). ELISAs using purified recombinant proteins have been widely used for routine diagnostic screenings and seropidemiological surveys (Jacobs et al., 1999; Pietkiewicz et al., 2004). However, the test is expensive, complicated, needs specialized laboratory facilities, and cannot be used in the field. Meanwhile, the ICT is fast and does not require costly equipment and expertise (Huang et al., 2004; Terkawi et al., 2013). The use of recombinant antigens for *T. gondii* serodiagnosis provide better and easier test standardization with fewer production costs over parasite lysate antigens (Pietkiewicz et al., 2004). To distinguish chronic from acute infections, immunoglobulin G (IgG) levels is compared with IgM and/or IgA levels (Jacobs et al., 1999). However, IgM antibodies can persist for months or years after initial infection with *T. gondii*; thus, it cannot be used as an indicator of acute infection (Fuccillo et al., 1987). Nevertheless, there are antigens that can be used to differentiate between the two infection stages.

The dense granules of *T. gondii* play a critical role in the host cell invasion, maintenance of the parasitophorous vacuole (PV), and parasite survival following cell invasion (Cesborn, 1994). These organelles then secrete dense granule antigens (TgGRAs), including GRA7, which become vital components of the PV and PV membrane surrounding the tachyzoites and in the cytoplasm and cyst wall of bradyzoites (Cesborn, 1994; Bonhomme et al., 1998; Nam, 2009). The potential of TgGRA7 as a serodiagnostic marker for toxoplasmosis in humans has already been confirmed using indirect ELISA, with a sensitivity of 81 to 98.9% and specificity of 98 to 100% (Jacobs et al., 1999; Kotresha et al., 2012; Felgner et al., 2015). It has also been reported to have higher detection rates as compared to other *T. gondii* antigens,

including the surface antigen (TgSAG1), rhoptry (TgROP1), and matrix antigens (TgMAG1), and TgGRA8 (Pfrepper et al., 2005; Kotresha et al., 2012). While TgGRA7 has been well utilized as an antigen for ELISA, only one report has documented its efficacy as an antigen for ICT. The ICT based on TgGRA7 was highly specific and sensitive for the detection of antibodies against *T. gondii* infection in pigs and were substantially concordant with the results of LAT and iELISA with TgGRA7 (Terkawi et al., 2013). However, there is no study yet documenting its use for serodiagnosis of human toxoplasmosis.

This present study aimed to assess the reliability of an ICT using TgGRA7 in the detection of *Toxoplasma* infections in humans by comparing the ICT results with standard iELISA and LAT tests. My findings revealed that the TgGRA7-ICT is a reliable test for the diagnosis of human toxoplasmosis, producing results similar to conventional serological methods.

2-2. Materials and Methods

Ethical clearance and consent

The study was performed in accordance with the principles of the Declaration of Helsinki. Ethical clearance and approval were obtained from the Institutional Review Board of the University of the Visayas, Cebu (UV-IRB2017-91) and Obihiro University of Agriculture and Veterinary Medicine (2017-05). Written informed consent was obtained from each respondent before blood collection.

Human serum samples

In this study, we tested 88 human sera sampled in Cebu, Philippines from September 2016 to April 2018, which were aseptically collected by a licensed phlebotomist and separated by centrifugation and stored at -20°C until further use.

Preparation of recombinant TgGRA7

Total RNA from pelleted RH strain of *T. gondii* was isolated and cDNA was synthesized as previously described (Terkawi et al., 2013). The cDNA was used as a template for PCR amplification of the coding region of TgGRA7 (accession number JX045574.1, corresponding to amino acids 27 to 236) using specific primers that included a *Bam*HI restriction enzyme in the forward primer and an *Eco*RI site in the reverse primer. The PCR product was digested with *Bam*HI and *Eco*RI before in-frame ligation into the glutathione S-transferase (GST) fusion protein sequence of the *E. coli* expression vector pGEX-4T1 (GE Healthcare, Buckinghamshire, UK), which had been digested with the same set of restriction enzymes. The plasmid nucleotide sequence was determined using an ABI 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA). The recombinant protein of TgGRA7 (rTGRA7) was expressed as a GST fusion protein in the *E. coli* DH5α strain (Takara Bio, Inc., Shiga, Japan). The GST tag of the rTGRA7 was removed with thrombin protease (GE Healthcare) according to the manufacturer's instructions. The rTgGRA7 is a 29 kDa protein as confirmed through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie brilliant blue staining (MP Biomedicals Inc., Illkirch-

Graffenstaden, France). A bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA) was used to measure the protein concentration.

iELISA using commercial test

The Platelia Toxo IgG and IgM (Bio-Rad) tests are among the most widely used solid-phase enzyme-linked immunosorbent assays (ELISA) for the detection of anti-toxoplasmic IgG and IgM antibodies (Mouri et al., 2015; Mahinc et al., 2017). The results of both tests were expressed in international units per milliliter (IU/mL). For the detection of IgG using Platelia Toxo IgG, antibody titers ≥9 IU/mL were considered positive, ≥6 and <9 IU/mL were equivocal, while <6 IU/mL) were judged negative. As to Platelia Toxo IgM, the titers were considered positive if ≥1, equivocal if ≥0.8 and <1, and negative if <0.8. The tests were performed, and the results were interpreted according to the manufacturer's instructions.

iELISA using TgGRA7

The recombinant TGRA7 was diluted in 50 mM carbonate-bicarbonate buffer (pH 9.6) at a final concentration of 0.1 µM. Each well of the ELISA plate (Nunc, Denmark) was coated with 50 µL of the diluted antigen and incubated overnight at 4ºC. The subsequent steps were performed as previously described (Terkawi et al., 2013) except that the secondary antibodies used were goat HRP-conjugated anti-human IgG and IgM (Sigma-Aldrich, St. Louis, MO, USA) diluted at 1:60,000 and 1:10,000, respectively. The cutoff points were determined as the mean value at an optical density of 415 nm (OD_{415}) plus three standard deviations (0.109 for

IgM and 0.143 for IgG) of human sera (N = 10) previously tested negative using commercial LAT and ELISA kit.

Testing of sera using a latex agglutination test (LAT)

The LAT was performed based on the manufacturer's instructions (Toxocheck-MT; Eiken Chemical, Tokyo, Japan) and as previously described (Ybañez et al., 2019). Samples were considered positive when agglutination or visible clumping that spread throughout the well was observed.

Preparation of the ICT

The ICT strips were prepared as previously described (Terkawi et al., 2013; Fereig et al., 2018). Briefly, 1 mg/mL of the recombinant TgGRA7 was gently mixed with gold colloids (1:10, vol/vol) and incubated at room temperature (RT) for 20 min. Stabilization and blocking of the conjugate particles were done by adding 0.05% polyethylene glycol 20,000 (Sigma-Aldrich, St. Louis, MO, USA) and 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) to the mixture. The conjugated gold colloid was centrifuged at 18,000 × *g* for 30 min and the pellet was resuspended by sonication. It was washed with 0.05% PEG and 0.5% BSA in PBS and centrifuged again. The pellet or conjugate was diluted with an appropriate volume of solution containing 10 mM Tris-HCl (pH 8.2) and 5% sucrose, sprayed onto the glass fiber (Schleicher and Schuell Bioscience, Inc., Keene, NH, USA) and dried overnight at RT. The recombinant protein (1 mg/mL) and the purified rabbit anti-TgGRA7 IgG antibody (1 mg/mL) were linearly jetted onto a nitrocellulose membrane (Schleicher and Schuell) through a BioJet

3050 quanti-dispenser (BioDot, Inc., Irvine, CA, USA). After drying of the membrane at 50°C for 30 min, blocking was done using 0.5% casein in 50 mM boric acid buffer (pH 8.5). The membrane was then washed and air-dried overnight. The strips were then assembled on an adhesive card (Schleicher and Schuell) and then cut into 3-mm-wide strips using a BioDot cutter (BioDot Inc.). The assay was performed by pipetting 30 μL of undiluted human serum samples to the sample pad. The results were judged within 20 min of band appearance at the control and/or test line (Fig 4).

Statistical analyses

The sensitivity, specificity, and kappa values with a 95% confidence interval were calculated using an online statistical tool (http://vassarstats.net/). The strength of agreement was graded with kappa values of fair (0.21 to 0.40), moderate (0.41 to 0.60), substantial (0.61 to 0.80), and very good (0.81 to 1.00). The correlation coefficients between relative intensity in the ICT band and absorbance values in the ELISA were calculated using Pearson's correlation coefficient. To calculate the relative ICT band intensity, ICT pictures were converted into 8-bit JPEG images, then the intensity of the grayscale images was analyzed using ImageJ software v. 1.49 (Mac version of NIH Image, http//rsb.info.-nih.gov/nih-image/) as described (Fereig et al., 2018).

2-3. Results

The efficacy of the ICT I developed using the TgGRA7 was validated by testing 88 human serum samples, and the results were compared with the results of iELISA based on

TgGRA7, commercial iELISA (com iELISA), and LAT. Table 4 shows that ICT using TgGRA7 was able to detect a total of 27 positive samples, which is similar to the detection of the two iELISAs. Four samples were negative for ICT but positive by LAT. Moreover, TgGRA7-iELISA detected IgM antibodies from three serum samples, while only one sample was positive using TgGRA7-ICT. Nonetheless, using the two iELISAs as reference, my ICT had a sensitivity of 93.1-100% and specificity of 100% for the detection of IgG and IgM and/or IgG antibodies (Table 5). A sensitivity of 87.1% and specificity of 100% were obtained when LAT was used as a reference test. With kappa values ranging from 0.897 to 1 (Table 5), my TgGRA7-ICT results were in very good agreement with the results of the iELISAs and LAT. Furthermore, I assessed the correlation between the ICT band intensity and ELISA OD values using ImageJ software v. 1.49 (Fig 5). I found a strong correlation between the relative band intensity in the TgGRA7-ICT and absorbance values in the TgGRA7-iELISA (Pearson's r=0.7876).

2-4. Discussion

The TgGRA7-ICT displayed excellent diagnostic performance in the detection of anti-*Toxoplasma* antibodies in human serum samples, demonstrating high specificity and sensitivity, and very good agreement with the results of standard serodiagnostic tests. These results corroborated with previous studies showing that TgGRA7 is a useful serological marker for the detection of IgG in acute and chronic infections (Bonhomme et al., 1998; Jacobs et al., 1999). Moreover, it has been documented that IgG antibodies against TgGRA7 appeared significantly earlier than those against TgSAG1 and TgMAG1 in human sera (Pfrepper et al., 2005). Similarly, other reports have confirmed that TgGRA7 is more sensitive with serum

samples from recently and acutely infected individuals than with samples from chronic infections (Pietkiewicz et al., 2004; Kotresha et al., 2012; Selseleh et al., 2012). In this study, TgGRA7-iELISA successfully detected antibodies in the acute and chronic infection stages. However, the non-detection of IgM antibodies from two other positive serum samples by TgGRA7-ICT may due to the low antibody titer in the sera, which leads to the inability to capture adequate conjugated antigens to produce a colored reaction on the membrane. The TgGRA7 protein is expressed by all infectious stages of *T. gondii* and is abundant on the surface of host cells and within the PV lumen, the PVM, and the host cell cytosol (Cesborn, 1994; Bonhomme et al., 1998; Nam, 2009), which may explain its strong antigenic characteristics. Following the rupture of their host cells, the tachyzoites and bradyzoites release the TgGRA7, allowing the antigens to have direct contact with the host immune system (Jacobs et al., 1999; Selseleh et al., 2012). This eventually stimulates a strong antibody response in both the early and late stages of infection (Jacobs et al., 1999; Coppens et al., 2006).

Altogether, my data suggest that the current ICT with TgGRA7 is a reliable test for the diagnosis of human toxoplasmosis, producing results similar to conventional serological methods.

2-5. Summary

In the present study, I have succeeded in developing a highly sensitive and specific ICT based on TgGRA7 for the detection of antibodies against *T. gondii* infection in humans. Aside from its highly reliable serodiagnostic performance, my current ICT is convenient to use, rapid, affordable and is easy to standardize in comparison with the existing serological tests. Thus,

this can be used as a screening tool for routine testing of toxoplasmosis and a good option for point of care application. Furthermore, this study documents the first utilization of TgGRA7 as an antigen in ICT for serodiagnosis of toxoplasmosis in humans.

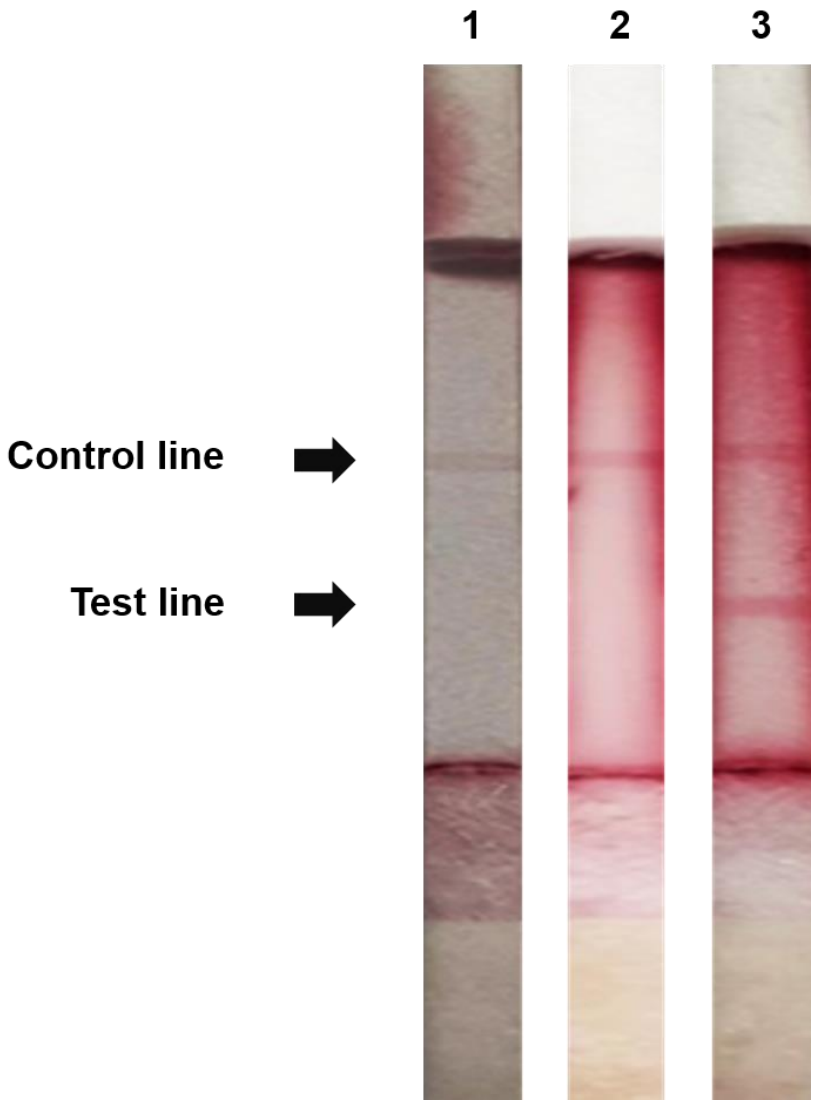


Fig 4. Representative images of the TgGRA7-ICT reactions. Strip 1: PBS, strip 2: *T. gondii*-negative serum, strip 3: *T. gondii*-positive serum.

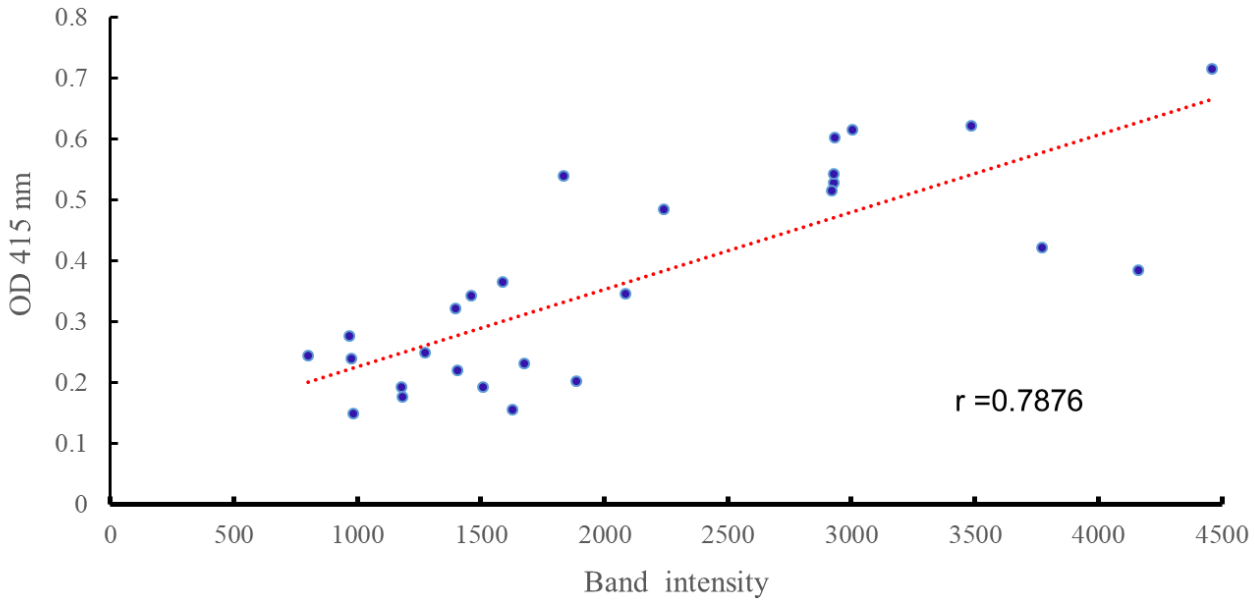


Fig 5. Correlation between ICT band intensity and iELISA OD values. Scatter plot shows the correlation between relative intensity in the ICT and absorbance values in the ELISA using human serum samples. The equation represents the approximation formula. The break line represents the calculated line of best fit. Correlation coefficients were calculated using Pearson's correlation coefficient: |r|=0.70, strong correlation; 0.5<|r|<0.7, moderately strong correlation; and |r|=0.3–0.5 weak to moderate correlation.

Table 4. Results of human sera tested by TgGRA7-ICT, -TgGRA7-iELISA, com-iELISA, and LAT (N=88).

TgGRA7-ICT	TgGRA7-iELISA				com-iELISA				LAT		TOTAL
	IgG		IgG and/or IgM		IgG		IgG and/or IgM				
	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	
Negative	61	0	59	2	61	0	59	2	57	4	61
Positive	0	27	0	27	0	27	0	27	0	27	27
Total	**61**	**27**	**59**	**29**	**61**	**27**	**59**	**29**	**57**	**31**	**88**

Table 5. Sensitivity and specificity of the TgGRA7-ICT in the detection of specific *T. gondii* antibodies in human sera compared with the results in the TgGRA7-iELISA, com-iELISA, and LAT.

Parameter	TgGRA7-iELISA		com-iELISA		LAT
	IgG	IgM &/or IgG	IgG	IgM &/or IgG	
Sensitivity (%)	100	93.1	100	93.1	87.1
Specificity (%)	100	100	100	100	100
Kappa Value	1	0.948	1	0.948	0.897

The parameters were calculated using an online statistical tool (www.vassarstats.net). The strength of agreement (kappa value) between each test and the ICT was graded as fair (0.21-0.40), moderate (0.41-0.60), substantial (0.61-0.80), and very good (>0.90).

Chapter 3

Detection of antibodies against *Toxoplasma gondii* in cats using an immunochromatographic test based on GRA7 antigen

3-1. Introduction

Toxoplasmosis is among the most common parasitic infections affecting humans and other warm-blooded animals and is caused by the intracellular apicomplexan parasite, *Toxoplasma gondii* (Hill et al., 2005). *T. gondii* reproduces in the gut of cats, its known definitive hosts (Dubey and Beattie, 1988). Oocysts are shed through the cat feces, which can be ingested by its intermediate hosts (Torrey and Yolken, 2013). Postnatally, *T. gondii* infection in humans is acquired by ingestion of raw meat infected with tissue cysts, consumption of food and drink contaminated with oocysts, or direct contact from the environment, such as the soil (Dubey, and Jones, 2008). Vertical transmission of the parasite through the placenta from the infected mother to the fetus may also occur (Paquet and Yudin, 2013).

T. gondii infection in cats is generally asymptomatic and latent in nature (Elmore, and Jones, 2010); however, it causes severe neurologic or ocular diseases in the fetus during pregnancy and in immunocompromised people (Dubey, and Jones, 2008). Moreover, cat ownership and frequent contact with cats have been identified as significant risk factors for *T. gondii* infection in humans. Being the definitive host of *T. gondii*, cats have a primary role in the transmission of the parasite, and frequent contact with cats increases the risk of infection (Chiang et al., 2012; Agmas et al., 2015; Ybañez et al., 2019). Therefore, the detection of *T. gondii* infection in cats is vital for diagnosis and control of toxoplasmosis.

Frequently used diagnostic tests of *Toxoplasma* infection in humans and animals are based on the serological detection of specific antibodies, such as the enzyme-linked immunosorbent assay (ELISA), and immunochromatographic test (ICT) (Hill and Dubey, 2002). ELISA using *Toxoplasma* lysate antigens (TLAs) has been used as a diagnostic method of *T. gondii* infection; however, ELISA based on purified recombinant proteins is preferably used for routine diagnostic screenings and seropidemiological surveys due to its easy test standardization and lesser production costs than TLAs (Jacobs et al., 1999; Pietkiewicz et al., 2004). Moreover, the use of ICT based on recombinant antigens has gained popularity as it is fast, easy to use, economical, and can be used in the field (Huang et al., 2004; Terkawi et al., 2013).

The dense granule antigen 7 of *T. gondii* (TgGRA7) is an essential component of the parasitophorous vacuole (PV) and PV membrane surrounding the tachyzoites and the cyst wall of the bradyzoites (Cesborn, 1994; Bonhomme et al., 1998; Nam, 2009). The effectiveness of TgGRA7 as a serodiagnostic marker for *T. gondii* infection have already been confirmed using iELISA, with a sensitivity of 81 to 98.9% and specificity of 98 to 100% (Jacobs et al., 1999; Kotresha et al., 2012; Felgner et al., 2015) in humans, and 94.9% sensitivity and 97.9% specificity in cats (Cai et al., 2015). Whereas TgGRA7 has been widely employed as antigen for ELISA, there is only one study that has documented its potential as antigen for ICT. Test results of the serological detection of *T. gondii* infection in pigs using ICT based on TgGRA7 were highly sensitive and specific and were substantially concordant with the results of latex agglutination test (LAT) and TgGRA7-based iELISA (Terkawi et al., 2013). There is no study yet reporting its potential as an antigen for ICT serodiagnosis of *T. gondii* infection in cats.

In this study, I evaluated the efficacy of an ICT using TgGRA7 in the detection of *Toxoplasma* infections in cats and compared the results with iELISAs using TgGRA7 and

lysate antigens of *T. gondii* strains, RH, PLK, and VEG. My results revealed that TgGRA7-ICT is a reliable test for the diagnosis of anti-*T. gondii* antibody in cats, producing comparable results as conventional serological methods.

3-2. Materials and methods

Ethical clearance

This study was performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology, Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments at Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan (permit number 19-3).

Cat serum samples

In this study, a total of 100 cat serum samples were collected from Animal Welfare and Management Center, Okinawa, Japan. The sera were stored at -30°C until further use.

Recombinant TgGRA7 preparation

Total RNA from the pelleted RH strain of *T. gondii* was isolated, and cDNA was synthesized and amplified as previously described (Terkawi et al., 2013). The recombinant protein of TgGRA7 (rTGRA7) was expressed as a glutathione S-transferase (GST) fusion protein in the *E. coli* DH5α strain (Takara Bio, Inc., Shiga, Japan). The GST tag of the rTGRA7 was cut using thrombin protease (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. The rTgGRA7 is a 29 kDa protein as

confirmed through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

T. gondii culture and lysate antigen preparation

T. gondii tachyzoites from the RH, PLK, and VEG strains were maintained in African green monkey kidney (Vero) cells cultured in Eagle's Minimum Essential Medium (EMEM, Sigma, St. Louis, MO, USA) supplemented with 8% heat-inactivated fetal bovine serum (FBS). For purification of tachyzoites, the infected cells were washed with cold phosphate-buffered saline (PBS). Cell pellets were resuspended in medium and passed through a 27-gauge needle and then through a 5.0-µm-pore filter (Millipore, Bedford, MA, U.S.A.). After centrifugation at 10,000 × g for 10 min at 4°C, the pellet was resuspended in cold PBS and sonicated at 3 × 30 sec at 5 kHz (Iwaki Ultrasonic Cleaner, Tokyo, Japan). The cells were freeze-thawed three times and centrifuged again. The supernatant was collected, and the concentration of the lysates was measured using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA). The TLAs were kept at -30°C until use.

iELISA using TgGRA7 and TLAs

The 96-well ELISA plates were coated overnight at 4°C with 50 µL of the recombinant TgGRA7 and the TLAs diluted to a final concentration of 0.1 µM and 2 µg/mL, respectively, using 50 mM carbonate-bicarbonate buffer (pH 9.6) as previously described (Selseleh et al., 2012; Abdelbaset et al., 2017). The cat sera were diluted at 1:500, and the secondary antibody used was horseradish peroxidase (HRP)-conjugated anti-cat IgG (Sigma-Aldrich, St. Louis, MO, USA) diluted at 1:4,000. The serum samples and secondary antibody were incubated for 1 hr at 37°C, while the subtrate was incubated at

RT for 1 hr. The cutoff points were determined as the mean value at an optical density of 415 nm (OD_{415}) plus three standard deviations (0.1738 for anti-TgGRA7, 0.1801 for anti-TgRH, 0.1462 for anti-TgPLK, and 0.1641 for anti-TgVEG) of cat sera (N = 24) previously tested negative using a commercial LAT kit (Toxocheck-MT; Eiken Chemical, Tokyo, Japan).

TgGRA7-ICT preparation

The ICT strips were prepared as described in Chapter 2 (Terkawi et al., 2013; Fereig et al., 2018). The recombinant TgGRA7 and purified rabbit anti-TgGRA7 IgG antibody (Terkawi et al., 2013) used were at a concentration of 1 mg/mL. The assay was performed by pipetting 30 μL of undiluted cat serum samples to the sample pad. The results were judged within 20 min of band appearance at the control and/or test line (Fig 6).

Statistical analyses

The sensitivity, specificity, and kappa values with a 95% confidence interval were calculated using an online statistical tool (http://vassarstats.net/). The strength of agreement was graded with kappa values of fair (0.21 to 0.40), moderate (0.41 to 0.60), substantial (0.61 to 0.80), and very good (0.81 to 1.00). The correlation coefficients between relative intensity in the ICT band and absorbance values in the ELISA were calculated using Pearson's correlation coefficient. To calculate the relative ICT band intensity, ICT pictures were converted into 8-bit JPEG images, then the intensity of the grayscale images was analyzed using ImageJ software v. 1.49 (Mac version of NIH Image, http//rsb.info.-nih.gov/nih-image/) as described (Fereig et al., 2018).

3-3. Results

In this study, we confirmed the effectiveness of the *Toxoplasma* ICT using TgGRA7 for serodiagnosis in cats by testing 100 cat serum samples. The results were compared with the results obtained using a commercial LAT kit, iELISA based on TgGRA7, and iELISAs using *T. gondii* RH, PLK, and VEG lysate antigens. Table 6 shows that my ICT using TgGRA7 detected a total of 74 positive samples, which is similar to the result obtained by iELISA using TgGRA7. There were two samples that tested positive by LAT but were negative for ICT. Moreover, 71 cat sera showed positive by iELISA using RH lysate, while 70 samples were positive using PLK and VEG lysates. Generally, my TgGRA7-ICT had a sensitivity of 97.4-100% and specificity of 89.7-100% for the detection of IgG antibodies (Table 7). Using TgGRA7-iELISA as a reference test, we obtained 100% sensitivity and specificity for my TgGRA7-ICT. When LAT was used as reference, my ICT showed a sensitivity of 97.4% and specificity of 100%. Meanwhile, using the lysate-based iELISAs, we obtained 100% and 87.7-89% sensitivity and specificity, respectively. With kappa values ranging from 0.901 to 1 (Table 7), the results of my TgGRA7-ICT were in very good agreement with the results of the different iELISAs and LAT. Furthermore, to confirm the reliability of test results using TgGRA7-ICT, the correlation between the ICT band intensity and ELISA OD values was analyzed (Fig 7). The analysis revealed a strong correlation between the relative band intensity in the TgGRA7-ICT and absorbance values in the GRA7-iELISA (Pearson's r=0.8957).

3-4. Discussion

Cats are important in the transmission of toxoplasmosis through the shedding of the infectious oocysts in their feces which may contaminate the environment. Serological

testing such as the ICT confirms infection in cats; thus, they can be brought to animal hospitals where appropriate medication can be started. As for seronegative cats, precautionary measures could be done to reduce the risk of infection including keeping the cats indoors to avoid hunting of possibly-infected rodents and other animals, avoiding raw or undercooked meat in the cat's diet, and disposing and cleaning cat litters regularly to minimize chances of exposure to infective oocysts and shedding the oocysts into the environment.

The TgGRA7-ICT developed in this study demonstrated an excellent diagnostic capability in the detection of anti-*Toxoplasma* antibodies in cat serum samples, showing high specificity and sensitivity, and very good agreement with the results of current serodiagnostic methods. These results were supported by previous studies showing the usefulness of TgGRA7 as a serological marker for the detection of IgG in acute and chronic infections in humans (Jacobs et al., 1999; Nam, 2009) and animals, including dogs (Wang et al., 2014) and cats (Cai et al., 2015). The strong antigenic characteristics of TgGRA7 could be due to its abundance on the surface of host cells and within the PV, the PVM, and the host cell cytosol and its expression in all infectious stages of *T. gondii* (Cesborn, 1994; Bonhomme et al., 1998; Nam, 2009). Direct contact of TgGRA7 with the host immune system following its release by the tachyzoites and bradyzoites after host cell rupture prompts a strong antibody response in both early and late stages of infection (Jacobs et al., 1999; Coppens et al., 2006; Selseleh et al., 2012).

Overall, the ICT results obtained in this study are similar to the results using conventional serological methods. This suggests that an ICT based on TgGRA7 can be used for serodiagnosis of *T. gondii* infection in cats.

3-5. Summary

In the present study, I was able to develop a highly reliable ICT based on TgGRA7 for the detection of antibodies against *T. gondii* infection in cats. The GRA7-ICT is fast, easy to use, affordable, and can be easily standardized in comparison with the current serological tests. Thus, this can be used as an alternative test for routine screening of *T. gondii* in cats. This study is the first report on the use of TgGRA7 as an ICT antigen for the serodiagnosis of *T. gondii* infection in cats.

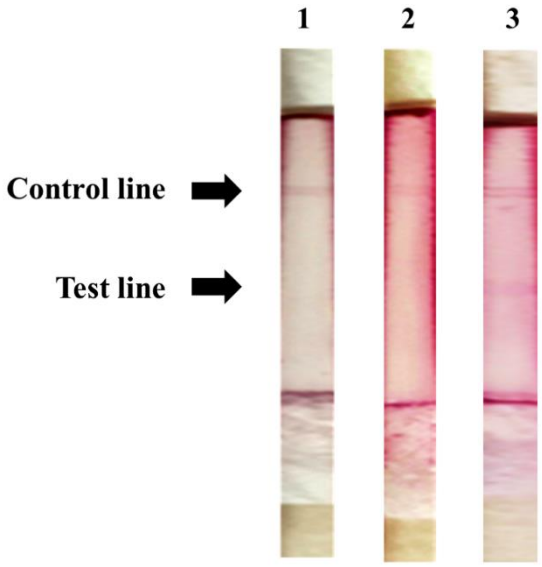


Fig 6. Representative images of the TgGRA7-ICT reactions. Strip 1: PBS, strip 2: *T. gondii*-negative cat serum, strip 3: *T. gondii*-positive cat serum.

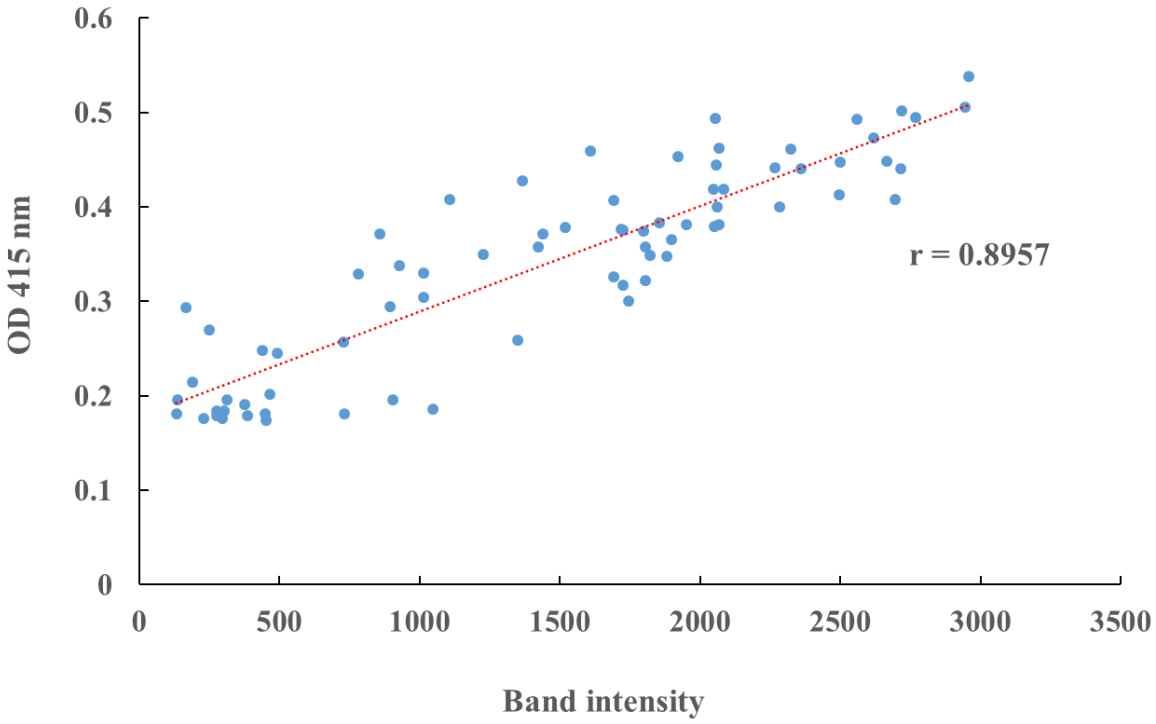


Fig 7. Correlation between ICT band intensity and iELISA OD values. Scatter plot shows the correlation between relative intensity in the ICT and absorbance values in the ELISA using cat serum samples. The break line represents the calculated line of best fit. Correlation coefficients were calculated using Pearson's correlation coefficient: |r|=0.70, strong correlation; 0.5<|r|<0.7, moderately strong correlation; and |r|=0.3–0.5 weak to moderate correlation.

Table 6. Results of cat sera tested by TgGRA7-ICT, TgGRA7-iELISA, TLA-iELISAs, and LAT (N=100).

TgGRA7-ICT	iELISA								LAT		TOTAL
	TgGRA7		TgRH		TgPLK		TgVEG				
	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	
Negative	26	0	26	0	26	0	26	0	24	2	26
Positive	0	74	3	71	4	70	4	70	0	74	74
Total	**26**	**74**	**29**	**71**	**30**	**70**	**30**	**70**	**24**	**76**	**100**

Table 7. Sensitivity and specificity of the TgGRA7-ICT in the detection of specific *T. gondii* antibodies in cat sera compared with the results of the TgGRA7-iELISA, TLA-iELISAs, and LAT.

Parameter	iELISA				LAT
	TgGRA7	TgRH	TgPLK	TgVEG	
Sensitivity (%)	100	100	100	100	97.4
Specificity (%)	100	89.7	87.7	87.7	100
Kappa Value	1	0.943	0.901	0.901	0.947

The parameters were calculated using an online statistical tool (www.vassarstats.net). The strength of agreement (kappa value) between each test and the ICT was graded as fair (0.21-0.40), moderate (0.41-0.60), substantial (0.61-0.80), and very good (>0.90).

General discussion

Toxoplasmosis is a widely distributed zoonotic infection caused by the obligate intracellular apicomplexan parasite *T. gondii*. It is mainly transmitted through the ingestion of oocysts shed by an infected cat acting as its definitive host. The key to effective control and treatment of toxoplasmosis is prompt and accurate detection of *T. gondii* infection. As direct isolation of the *T. gondii* parasite is often difficult, several serodiagnostic methods have been established, such as the SFDT, DAT/MAT, IHAT, LAT, IFAT, ELISA, ICT, and the WB. To effect large-scale assessment of the presence of *T. gondii* infection, the development of specific, reliable, and fast approaches for its serodiagnosis remains a challenge.

While several countries are aware of the disease and have already implemented routine screening for toxoplasmosis, some (including the Philippines) are still unaware of the current status of the infection due to lack of nationwide epidemiological studies. In Chapter 1 of this study, the epidemiological status of the seroprevalence of *T. gondii* in humans, pigs, and cats in Cebu, Philippines, was assessed using LAT. The overall seroprevalence of *T. gondii* in humans obtained was 26.4%, with a higher seropositivity rate among women (27%) than in men (25.5%). However, sex was not found to be significantly associated with seropositivity in the current or previous studies in the Philippines. The detection rate among pregnant women in this study was comparable to the previous results in pregnant women in Singapore (Wong et al., 2000), and Vietnam (Buchy et al., 2003), but slightly lower than in Thailand (Andiappan et al., 2014) and higher in Taiwan (Hung et al., 2015). But only Andiappan et al. (2014) reported risk factors, namely the occupation (as laborers) and drinking water source, as significantly associated with *Toxoplasma* seropositivity in pregnant women.

The age or sex of respondents was not significantly associated with seropositivity rates in this study, just like the previous findings in some areas of the Philippines (Auer et al., 1995; Salibay et al., 2008). However, Kawashima et al. (2000) noted that infection rates in the Philippines increased with age. This implied that *T. gondii* may infect any sex at any age, but the frequency of seropositivity is higher among older individuals, most likely due to more prolonged exposure to risk factors than younger individuals.

Significantly higher seroprevalence in rural and suburban areas than in urban areas was previously reported in the country (Kawashima and Kawabata, 2000; Salibay et al., 2008), which was consistent with the findings of this study. The socioeconomic status of the participants may have attributed to the variance among locations which may affect hygiene practices and the likelihood of ingestion of oocysts from water, crops, animal products and unpasteurized milk contaminated from oocysts shed by cats and contaminated soil (Kawashima and Kawabata, 2000; Ertug et al., 2005).

A higher seroprevalence among individuals with apparent association with cats was also observed in the Philippines (Salibay et al., 2008). The close association with or ownership of cats has been identified as a vital risk factor for human infection worldwide. Being the definitive host of *T. gondii*, cats have a primary role in the transmission of the parasite, and frequent contact with cats generally increases the risk of infection due to contact with oocysts in cat feces (Chiang et al., 2012; Agmas et al., 2015). Moreover, this study is the first to serologically detect *T. gondii* infection in pregnant women in the Philippines. Congenital infection is a crucial part of the disease burden in human *Toxoplasma* infections (Robert-Gangneux and Dardé, 2012). The reported serodetection of *Toxoplasma* in pregnant women in this study may have a potentially detrimental effect on the fetus due to the risk of congenital infection.

The serodetection rate observed in cats in this study is similar to the previous report in the northern Philippines (Advincula et al., 2010), but is lower than the reported seroprevalence in Vietnam (Hosono et al., 2009), and higher than in Japan (Oi et al., 2015). Male cats were found to be more susceptible to infection (Advincula et al., 2010); however, other reports, including the current study, concluded that sex is not a risk factor for toxoplasmosis in cats (Smielewska-Loś and Pacoń, 2002; Gauss et al., 2003). The greater seropositivity observed in fertile cats in this study may be due to the behavioral habits of fertile cats to roam in search of a mate, which predisposes them to contact with oocyst-contaminated environment, infected rodents or other animals. Poor body condition was also reported to affect seropositivity in cats. It consequently leads to weakened immunity, thus increasing vulnerability to disease (Castillo-Morales et al., 2012; Tehrani-Sharif et al., 2015). However, body condition showed no significant association with seropositivity in cats in this study. Furthermore, the current study showed that the use of litter trays increased the likelihood of cats to become infected with *T. gondii*. Exposure to cat litter trays may facilitate contact with infective *T. gondii* oocysts, according to the Center for Disease Control. Seropositivity for infection in cats was significantly higher in suburban than in urban areas in this study. Generally, location (urban, suburban and rural areas) (Lopes et al., 2008; Oi et al., 2015), diet (Lopes et al., 2008; Opsteegh et al., 2012) and domestication (Lee et al., 2010; Opsteegh et al., 2012; Garcia et al., 2014; Guy and Penuliar, 2016) influence seropositivity in animals.

The present study also investigated the current infection status in swine in Cebu, Philippines, since its first report in slaughter pigs in 1982 (Manuel, 1982). Although the rearing system was not significantly associated with seropositivity in pigs in this study, the method by which pigs are farmed affects seroprevalence by potentially predisposing the animals to infection (Dubey, 2009; Guo et al., 2015). The substantially higher serodetection

rate among pigs farmed by extensive (non-intensive/backyard) production methods compared with intensively farmed pigs is due to the access of these animals to the outdoors, which increases contact with infected rodents and dead animals and oocyst-contaminated feed, water, and surroundings, as reported in the USA and Japan (Dubey and Beattie, 1988; Dubey and Jones, 2008; Guo et al., 2015). Access of cats to farm animals such as pigs has also been identified as a critical risk factor for the transmission of *T. gondii* infection (Herrero et al., 2016). The serological detection of *T. gondii* in pigs in Cebu more than three decades after its first detection revealed the constant presence of *T. gondii* in the area.

The prevalence rates obtained in this study were dependent on the detection method used. The LAT detects total antibody and does not distinguish between recent and past exposures. Because the performance of assays can differ, relative seroprevalence rates may also vary depending on the test used. Thus, statistical correlations on risk factors may also give varying results depending on the assay used in a study. It must be emphasized though that the LAT method used in this study had been used in several serological surveys with various animal species, including humans because of its ease of use, low cost, and high sensitivity, and specificity (Salibay et al., 2008; Matsuo et al., 2014). While LAT is widely used and considered to be reliable, its conduct can be time-consuming, which may necessitate the development of faster but equally reliable diagnostic tools, like the ICT.

In the succeeding chapters, the usefulness of TgGRA7-ICT was explored. In Chapter 2, the efficacy of the developed TgGRA7-ICT was validated by testing 88 human sera. Its results were compared with those obtained by ELISA based on TgGRA7, a commercial ELISA (Platelia Toxo IgG and IgM; Bio-Rad), and LAT (Toxocheck-MT; Eiken Chemical). The TgGRA7-ICT results revealed an excellent agreement with standard test results, as shown in its high sensitivity, specificity, and kappa values. A strong correlation between the relative ICT band intensity and absorbance values in the ELISA

was also obtained. Similarly, in Chapter 3, the developed TgGRA7-ICT was assessed for the detection of *Toxoplasma* infections in 100 cats. Its results were compared with that of the iELISAs using TgGRA7 and lysate antigens of *T. gondii* strains, RH, PLK, and VEG. The high sensitivity, specificity, and kappa values obtained in this study revealed that TgGRA7-ICT is a reliable test for the diagnosis of anti-*T. gondii* antibodies in cats, which produced comparable results with the conventional serological methods.

Chapters 2 and 3 showed that the developed TgGRA7-ICT demonstrated excellent diagnostic performance in the detection of anti-*Toxoplasma* antibodies in human and cat sera, showing high specificity and sensitivity, and very good agreement with the results of current serodiagnostic methods. These findings corroborate with previous studies that showed the usefulness of TgGRA7 as a serological marker for the detection of IgG in acute and chronic infections in humans (Bonhomme et al., 1998; Jacobs et al., 1999; Nam, 2009) and animals, including dogs (Wang et al., 2014) and cats (Cai et al., 2015). The strong antigenic characteristics of TgGRA7 could be due to its abundance on the surface of host cells and within the PV, the PVM, and the host cell cytosol and its expression in all infectious stages of *T. gondii* (Cesborn, 1994; Bonhomme et al., 1998; Nam, 2009). Following the rupture of the host cell, the TgGRA7 proteins are released from the tachyzoites and bradyzoites, allowing direct contact of the antigens with the host immune system. This eventually prompts a strong antibody response in both early and late stages of infection (Jacobs et al., 1999; Coppens et al., 2006; Selseleh et al., 2012).

Cats are vital in the transmission of toxoplasmosis through the shedding of the infectious oocysts in their feces, which may contaminate the environment leading to human infection. As the present study detected seropositive cats in areas with confirmed cases of seropositive humans, there is a need to conduct further studies in stray cats that are commonly found in areas selling street foods in the Philippines. The mode of transmission

is possibly resulting from the consumption of infected rats or meat. Moreover, seropositivity was still detected among pigs, which suggests the endemicity of *T. gondii* infection in pigs reared in Cebu. This poses a public health hazard to predominantly meat-eating Filipino people as *T. gondii* cysts in pork may be infectious if the meat is consumed undercooked.

Although serological evidence of exposure of pigs, humans, and cats to *T. gondii* was presented, my present study could not account for the status of the disease across the entire country because of sampling limitations. Therefore, the developed TgGRA7-ICT could be a helpful tool for future studies with an increased sampling size and area to clarify the epidemiological status of *T. gondii* in the Philippines. This serodiagnostic assay could also be useful for routine testing in cats and vulnerable populations, including pregnant women, immunocompromised individuals, and those exposed to cats.

General summary

Toxoplasma gondii is a ubiquitous single-celled intracellular apicomplexan parasite that causes toxoplasmosis, one of the world's most common parasitic diseases. Cats are the only known definitive hosts of *T. gondii,* but it can infect humans and practically all warm-blooded animals. Its public health importance is recognized worldwide because of its zoonotic potential to cause human abortion, stillbirth, and fetal abnormalities. It has been associated with mental and behavioral changes in humans. Acute infection of *T. gondii* is also potentially fatal in immunocompromised individuals. Considering the health threat it poses, assessing its presence using the appropriate diagnostic tools can be the first step in determining its relevance to a locality for proper intervention.

In Chapter 1, the *Toxoplasma* seroprevalence in pigs, humans, and cats in Cebu, Philippines, was evaluated. *T. gondii* infection was reported to be detected in pigs about three decades ago. A total of 924 humans, 104 cats, and 514 slaughter pigs were tested for antibodies against *T. gondii* using a commercial latex agglutination test. The results revealed positive detection rates of 26.3% (244/924) for humans, 42.3% (44/104) for cats, and 13.4% (69/514) for slaughter pigs. Statistical analyses revealed that the area *(P* = 0.004), cat ownership (*P* = 0.020), the frequency of contact with cats (*P* < 0.0001), and consumption of street foods (*P* = 0.043) were significantly associated with seropositivity for *T. gondii* in humans. Meanwhile, the use of litter trays (*P* = 0.001) and contact with other animals (*P* = 0.007) were significantly associated with seropositivity in cats. The odds ratio for selected significant factors revealed that living in suburban areas (OR 1.66, 95% CI: 1.20–2.31), owning a cat (OR 1.482, 95% CI: 1.07–2.07) and eating street foods (OR 1.585, 95% CI: 1.01–2.48) were associated with an increased risk of *T. gondii* exposure in humans. In cats, the use of a litter tray (OR 4.5, 95% CI: 1.73–11.71) was associated with

an increased risk of exposure. None of the profile parameters were found to be significantly associated with seropositivity in slaughter pigs ($P > 0.05$). This study reports the first serological detection of *T. gondii* in humans and cats in Cebu, Philippines, and the first assessment of the prevalence of the parasite in pigs in the area since its initial detection in 1982. This is also the first report documenting the seropositivity of *T. gondii* in pregnant women in the country. The confirmed seropositivity of *T. gondii* in Cebu, Philippines, in this study implies the endemicity of toxoplasmosis in this area and highlights the need for routine testing and increased public awareness.

T. gondii secretes huge quantities of dense granule antigens (TgGRAs), which are fundamental to the survival of the parasite. The TgGRA7 is found abundantly on the surface and cytosol of host cells, and within the PV lumen and membrane. It stimulates a strong antibody response during acute and chronic infections. While it has been well utilized as an antigen for ELISA, only one report has documented its efficacy as an antigen for ICT in pigs. There is no study yet documenting its use for ICT serodiagnosis of *T. gondii* infection in humans and cats. Hence, the corresponding potential of TgGRA7 as an antigen was evaluated in the proceeding studies.

In Chapter 2, the efficacy of the developed TgGRA7-ICT was validated by testing 88 human sera. Its results were compared with those obtained by ELISA based on TgGRA7, a commercial ELISA (Platelia Toxo IgG and IgM; Bio-Rad), and LAT (Toxocheck-MT; Eiken Chemical). The TgGRA7-ICT results revealed an excellent agreement with standard test results, as shown in its high sensitivity, specificity, and kappa values. A strong correlation between the relative ICT band intensity and absorbance values in the ELISA was also obtained. Altogether, the data suggest that the current ICT with TgGRA7 is a reliable test for the diagnosis of human toxoplasmosis, which produced results similar to conventional serological methods. Thus, this can be used as a screening tool for routine

testing of toxoplasmosis and a good option for point-of-care application. This study is the first report on the use of TgGRA7 as an ICT antigen for the serodiagnosis of human toxoplasmosis.

In Chapter 3, the usefulness of theTgGRA7-ICT was assessed for the detection of *Toxoplasma* infections in 100 cats. Its results were compared with that of the iELISAs using TgGRA7 and lysate antigens of *T. gondii* strains, RH, PLK, and VEG. The high sensitivity, specificity, and kappa values obtained in this study revealed that TgGRA7-ICT is a reliable test for the diagnosis of anti-*T. gondii* antibodies in cats, which produced comparable results with the conventional serological methods. This study is the first report on the use of TgGRA7 as an ICT antigen for the serodiagnosis of *T. gondii* infection in cats.

The ultimate goal of this endeavor was to develop *T. gondii* infection serodiagnostic assays that can be suitable for a nationwide epidemiological and point-of-care application. This goal was achieved by conducting human and animal epidemiological studies using current serodiagnostic tools to establish baseline information and identify associated risk factors for intervention in the Philippines, and by evaluating the application of TgGRA7 as antigen for *T. gondii* serodiagnosis using ICT in humans and cats. The approach adopted in this research supports the development of TgGRA7 for ICT in assessing *T. gondii* infection on a large scale.

和文要約

トキソプラズマ症は世界で最も拡散している寄生虫病の一つである。本症はアピコンプレックス門に属する細胞内寄生原虫であるトキソプラズマ・ゴンディィにより引き起こされる。トキソプラズマはヒトを含めた様々な温血動物に感染でき、感染動物の組織に存在するブラディゾイトを含んだシスト、あるいは終宿主のネコ科動物から排泄されたオーシストの摂取により伝搬する。トキソプラズマは世界人口の 1/3 に感染しているが、一般的に健常者は感染に対し不顕性で特異的な臨床症状はなく、稀にインフルエンザ様の症状を呈する。トキソプラズマ症は流産、死産、胎児異常を引き起こす人獣共通感染症であることから、世界中で公衆衛生的な重要性が認知されている。また、本症は精神・神経疾患や行動様式に関連することが示されている。トキソプラズマの急性感染は HIV 患者などの免疫不全の状態では重篤化するリスクがある。トキソプラズマ症による健康障害を考慮すると、迅速で正確な感染の検出がきわめて重要である。適切な診断方法を用いた感染状況の把握は、現場での最善な対応を判断するための第一段階となる。

第一章では、フィリピン・セブにおけるヒト、ブタ、ネコのトキソプラズマに対する血清疫学調査を行った。セブにおけるトキソプラズマ感染は３０年前にブタで報告されている。ヒト 924 検体、ネコ 104 検体、ブタ 514 検体について、市販ラテックス凝集試験（LAT）を用いてトキソプラズマの抗体検査を行った。抗体陽性率は、ヒト 26.3% (244/924)、ネコ 42.3% (44/104)、ブタ 13.4% (69/514)となった。統計解析により、居住地域（$P = 0.004$)、ネコの所有（$P = 0.020$)、ネコとの接触頻度（$P < 0.0001$)、露店で売られている食べ物の消費（P

= 0.043) がヒトにおけるトキソプラズマの抗体陽性に関与していることが明らかとなった。一方、ネコ用トイレの使用 (P = 0.001)、他の動物との接触 (P = 0.007) がネコにおけるトキソプラズマの抗体陽性に関連していた。ヒトでの上記感染要因を用いたオッズ比は、郊外での居住で 1.66 (95% CI: 1.20-2.31)、ネコの所有で 1.482 (95% CI: 1.07-2.07)、露店で売られている食べ物の消費で 1.585 (95% CI: 1.01-2.48)であった。ネコの場合、オッズ比はネコ用トイレの使用で 4.5 (95% CI: 1.73-11.71)となった。ブタにおける抗体陽性に関連する要因は検出されなかった。本研究はフィリピン・セブにおいてヒトとネコにおけるトキソプラズマ抗体陽性例の最初の報告となる。またブタでは、1982 年の報告以来、最初のトキソプラズマ抗体陽性例である。私の知る限りでは、妊娠女性におけるトキソプラズマ抗体陽性例もフィリピンで最初の報告である。フィリピン、セブにおけるトキソプラズマ抗体陽性例の確認は調査地域における地方的流行を示唆しており、日常的な検査と啓発が必要とされる。

トキソプラズマはその生存に重要なデンスグラニュル抗原 (TgGRA) を多量に分泌する。TgGRA7 は宿主細胞の細胞表面と細胞質、寄生胞の内腔と膜に多量に存在している。TgGRA7 はタキゾイトとブラディゾイトで発現しており、宿主細胞の破壊後に細胞外へ放出され、宿主免疫系に直接曝される。最終的に、この抗原の動態は急性及び慢性感染期に強力な抗体応答を刺激することになる。TgGRA7 は ELISA で頻繁に使用されているが、イムノクロマトテスト (ICT) ではブタで使用された報告が一つあるのみである。これまでのところ、ヒトとネコのトキソプラズマ感染では TgGRA7 を使用した ICT の研究報告はない。そのため、続く研究にて TgGRA7 搭載 ICT の可能性を評価した。

第二章では、ヒト血清 88 検体を用いて TgGRA7 搭載 ICT の効果を検証した。得られた結果は、TgGRA7 搭載 ELISA、市販 ELISA 及び LAT で得られた結果と比較した。TgGRA7 搭載 ICT の結果は、感度、特異性、κ値を解析して標準試験の結果と高い一致度を示した。ICT で現れたバンド強度と ELISA の吸光度の間には強い正の相関関係が認められた。以上より、TgGRA7 搭載 ICT は従来の血清診断法と同様の結果が得られたため、ヒトのトキソプラズマ症の診断に理想的な検査方法であることが示唆された。従って、TgGRA7 搭載 ICT はトキソプラズマ症の日常的な検査のためのスクリーニング手法及びポイント・オブ・ケアに用いられる選択肢の一つとして使用できる。本研究はヒトのトキソプラズマ症の血清診断のために TgGRA7 を抗原として利用した ICT の最初の報告となる。

第三章では、ネコ血清 100 検体を用いて TgGRA7 搭載 ICT の効果を検証した。ICT の結果は、TgGRA7、トキソプラズマ株 RH、PLK、VEG のライセート抗原を搭載した ELISA の結果と比較した。感度、特異性、κ値を判断して ELISA の結果と高い一致度を示したことから、TgGRA7 搭載 ICT はネコのトキソプラズマ抗体の検出に適した検査方法であることが明らかとなった。本研究はネコのトキソプラズマ感染の血清診断のための ICT 用抗原として TgGRA7 を使用した最初の報告となる。

本研究の最終目標は、国レベルの疫学調査とポイント・オブ・ケアに適したトキソプラズマ感染用血清診断法の開発であった。現在の血清診断法を用いたヒトと動物の疫学調査により診断基準とフィリピンでの対策のためのリスク要因を同定した。また、ヒトとネコで TgGRA7 搭載 ICT の有効性を評価することにより、本研究目標は達成された。本研究での取り組みはトキソプラズマ感染の大規模な調査に TgGRA7 搭載 ICT の活用を指示するものとなる。

Acknowledgments

The success and outcome of this research endeavor would not have been possible without the guidance and assistance from many people, whom I am incredibly privileged to have worked with.

First, I would like to give my respect and gratitude to Prof. Yoshifumi Nishikawa, my main supervisor, for giving me the excellent opportunity to complete my Ph.D. degree under his supervision. It is truly an honor. Thank you very much, Sensei, for all the advice, ideas, patience, moral support, understanding, and guidance throughout this project. More than your knowledge, it is your enthusiasm, dedication, and work ethics that inspired me. I am always indebted to you for believing in me and for giving me the chance to grow in this field of research. You have mentored me every step of the way. I couldn't thank you enough for everything that you did for me.

I am fortunate and thankful to have Prof. Xuenan Xuan and Prof. Makoto Igarashi as my co-supervisors, and Prof Shin-ichiro Kawazu and Prof. Naoaki Yokoyama as members of my thesis committee. Their advice and useful comments have helped improve my research work and my thesis. I am also thankful to Prof. Kawazu and Dr. Keisuke Suganuma for giving me access and for allowing me to use their lab facilities for my experiments. Similarly, my gratitude goes to all the other professors, post-doc researchers, students, and staff of the National Research Center for Protozoan Diseases (NRCPD) especially to Mr. Tsuyoshi Habaguchi, Ms. Ai Shindo, and Ms. Miki Fukunishi, for all their guidance and kind assistance, and for making my stay at the center memorable and worthwhile.

I owe my deep gratitude to the Obihiro University of Agriculture and Veterinary Medicine (OUAVM) for the financial and academic support provided to me. Had it not for my scholarship, I wouldn't have attained my Ph.D. degree. My special acknowledgment to Mr. Hiroshi Ishiyama and the rest of the staff of the Admissions and Educational Affairs Section, Ms. Mizuki Chiba, Ms. Yuki Hara and Ms. Yukiko Hattori of the International Student Office, for all their unwavering support and assistance since day one of my life as a student of OUAVM.

I sincerely thank all our research collaborators in the Philippines; Dr. Adrian Ybañez, Dr. Gloria Delan, and Dr. Jorim Langbid of the Cebu Technological University, Dr. Paul John Geraldino of the University of San Carlos, Dr. Johanne Nuevarez of the UV Gullas College of Medicine, Ms. Chadinne Girlani Busmeon and Ms. Alexa Renee Viernes, my former students at the University of the Philippines Cebu, and Dr. Khalil Latorza of the Talisay City Slaughterhouse for kindly assisting me during the sampling and testing in the Philippines, and our collaborator in Japan, Dr. Hisako Kyan of the Okinawa Prefectural Institute of Health and Environment for generously providing me with the cat serum samples I used in my experiment.

It is a great honor for me to be a part of the Host Defense (HD) laboratory. And I owe a lot to all my labmates who have supported and guided me since the beginning. Special mention to Dr. Kousuke Umeda, Dr. Fumiaki Ihara, Dr. Ragab Fereig, Dr. Naomi Shimoda, Dr. Mingming Liu, and Dr. Paul Franck Adjou Moumouni for providing all the help and guidance that I need until the completion of my research work. To Dr. Hanan Abdelbaky and Dr. Pagmadulam Baldorj who are more than just labmates to me. Thank you, my sisters, for always being there for me in any way you can, for the memories of hard work, of encouraging each other, and for the great friendship.

To my dear friends, Dr. Afraa Elata, Dr. Minh Anh Dang Trinh, Dr. Vernadyn Morillo, Ms. Maria Agnes Tumwebaze, Ms. Eloiza May Galon, Dr. Mariani Fiorenza, and Dr. Joemark Narsico, thank you for going through hard times with me, for cheering me on, and for celebrating every accomplishment with me. I don't know how I would have survived my life in Japan without you. My profound gratitude also goes to Mrs. Onishi for going out of her way to help and make sure that my life away from home is comfortable and bearable.

I am grateful to my parents-in-law, Dr. Rufino and Mrs. Venus Ybañez, and my siblings-in-law for their unfailing love and support primarily to my family while I am away. My heartfelt gratitude to my siblings, Mrs. Yvette Myla Vacal, Mrs. Verna Ellenor Constantino, and Mr. Lix Dembo Daclan and their families, to Ms. Rosita Miñoza (Ate Des), and to all my relatives and friends back home, for their love, encouragement, advice, generosity, and prayers despite the long distance between us. I love you all.

To my Papa and Mama, Dr. Felix and Mrs. Cherrie Pie Palermo Daclan, I owe you my life and everything that I am now. Thank you for all your sacrifices and unconditional love while we were growing up. I love you both. I hope I made you proud there in heaven.

I give my whole heart in gratitude to my husband, Dr. Adrian Ybañez. It was a great comfort and relief to know that you are there to take care of our kids while I am away. I know it was difficult for you to take two roles at the same time, but you gladly did just for me to pursue my dream of attaining a Ph.D. degree. I appreciate you, Dad, every day for your undying love and for everything that you do for our family and me. To my children, Annika Rheece, Andrea Rikki, and Alexander Rufino Ybañez, you are my most precious achievement in life. You and Daddy are the big reason why I remain strong, despite all the trials that came my way. Your regular dose of love, encouragement, and support has fueled

me to get through all the tough times, and the daily struggle of being miles away from you. I love you all a million times.

Finally, I give my earnest gratitude to the Lord God Almighty for giving me the wisdom, the strength and determination to carry on with my studies and endure all the difficulties I encountered throughout this journey. Several times I felt weak and hopeless, but you were always there to strengthen me and give me hope. Thank you for your constant mercy and grace. I owe everything that I have accomplished to You, my Lord. To You, Oh God, I give all the glory and honor forever.

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