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Epidemiology of *Toxoplasma gondii* in Thailand

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“Never compare yourself or others to other people. Everyone has their own struggles, own fights, and a different path that they choose to get to where they are. Everyone is who they are for a reason.”

“Never give up on something you really want. However impossible things may seem. There’s always a way.”

“Experience tells you what to do; Confidence allows you to do it.”

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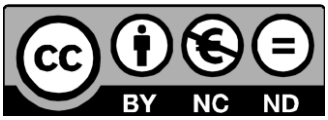

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Abbreviations

AS primer: Anti sense primer

BABS: Bovine albumin-buffered saline

bp: base pair

BSA: bovine serum albumin

CMAT: Commercial Modified Agglutination Test

Ct: Cycle threshold

DABCO: Diazabicyclo octane

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

FCS: Fetal Calf serum

HCl: Hydrochloric acid

IFAT: Indirect Immunofluorescence Antibody Test

IgG: Immunoglobulin G

IgM: Immunoglobulin M

IHAT: Indirect Hemagglutination Test

IMAT: In-house Modified Agglutination Test

LAT: Latex Agglutination Test

MAT: Modified Agglutination Test

$MgCl_2$: Magnesium chloride

MS markers: Microsatellite markers

PBS: Phosphate buffered saline

PCR: Polymerase Chain Reaction

PCR-RFLP: Polymerase Chain Reaction - Restriction Fragment Length Polymorphism

RNA: Ribonucleic acid

ROC: Receiver operating characteristics

RPMI: Roswell Park Memorial Institute medium

SE primer: Sense primer

UNG: Uracil DNA Glycosylase

Introduction

Toxoplasma gondii is an intracellular cosmopolitan parasite with a variable prevalence rate worldwide. It is estimated that *T.gondii* infects one third of world human population (Weiss and Dubey, 2009). This protozoan, included in Apicomplexa phylum, is one of the most common parasites. Prevalence of human infections by *T. gondii* varies around the world depending on several sociogeographical factors. The sources of infection could widely vary between different ethnic groups and different geographical locations. Acquisition of *T.gondii* infection is commonly acquired by the ingestion of tissue cysts, containing bradyzoites or by the ingestion of oocysts containing sporozoites. Sporozoites are the product of a sexual cycle from cat intestines (Tenter et al., 2000). This parasite presents three main stages, tachyzoites, bradyzoites and sporozoites (Dubey et al., 1998). Domestic and wild felids are the definitive hosts for this parasite.

The infection of *T. gondii* has been studied worldwide using various serological tests to reveal the presence of antibodies to *T. gondii* in humans as well as in animals (domestic animals and wildlife). However, there is no reference test to screen the infection with *T. gondii* in animal. Modified Agglutination Test (MAT) is the current serological screening test. It is the most adapted to many species (Dubey, 1997; Dubey, 2010b; Dubey at al., 2015). But, other serological tests such as IFAT, LAT, ELISA and IHAT are performed for seroprevalence study.

When focusing on Southeast Asian Region, contrasted results were observed with a high seroprevalence of human infection observed in some equatorial zones, up to 70% of seropositive in general inhabitants in Jakarta, Indonesia (Terazawa et al., 2003) or, 59.7% of inhabitants in Malaysia (Ahmad et al., 2014), while a low seroprevalence was observed in countries with tropical monsoon climate as Thailand, Vietnam, Laos and Cambodia. The equatorial zone should present factors supporting the circulation of *T. gondii*. Other risk factors as dietary habits, environmental conditions, socioeconomic level, contact with cats and hygiene are known to be involved in the propagation of *T. gondii*. But the reasons explaining these contrasted seroprevalence rates and particularly, the low prevalence in many Asian countries, as in Thailand, are still poorly understood.

Many *Toxoplasma gondii* strains have been isolated and genetically characterized in Europe, in North and South America, and to a less extent in Africa and China. These studies on genetic characterization have shown a global population structure of *T. gondii* with a

geographical distribution of the different genetic groups across the continents. But there is a lack of information on strains circulating in Southeast Asia.

Through this thesis work, we wanted, after a literature review on the epidemiology of *T. gondii* infection in human and animals in Southeast Asian countries, and on available data on *Toxoplasma* genotypes circulating in Asia, to contribute to the knowledge on *T. gondii* epidemiology in Thailand.

Field studies were conducted in Thailand to:

- Determine the seroprevalence of *T. gondii* in free-range chickens from 2 rural villages, Kangpralom and Wangpow of Kanchanaburi province, Thailand.

- Isolate *T. gondii* strains from seropositive animals and identify *T. gondii* genotypes by genetic characterization method with 15 microsatellite markers.

Chapter I: Generality on *Toxoplasma gondii*

I. Taxonomy of *T. gondii*

Table 1: Taxonomy of *Toxoplasma gondii*.

Taxonomy of *Toxoplasma gondii*	
Kingdom	Protista
Phylum	Apicomplexa
Class	Sporozoasida
Order	Eucoccidiorida
Family	Sarcocystidae
Genus	*Toxoplasma*
Species	*gondii*

II. Description of the parasite and its stages of development

Toxoplasma gondii is a cosmopolitan parasite with a variable frequency rate worldwide. It is estimated that *T.gondii* infects one third of world population (Weiss and Dubey, 2009). This protozoan is one of the most common parasites, which infects human and other warm-blooded animals. *Toxoplasma gondii* is an obligate intracellular parasite and it is included in Apicomplexa Phylum. Infections by *T. gondii* rates vary around the world depending on several sociogeographical factors. The sources of infection could vary greatly between different ethnic groups and different geographical locations. Acquisition of *T.gondii* infection is commonly acquired by the ingestion of tissue cysts that contain bradyzoites or by the ingestion of oocysts containing sporozoites. Sporozoites are the product of a sexual cycle in cat intestines (Tenter et al., 2000). This parasite presents three main stages, tachyzoites, bradyzoites and sporozoites (Dubey et al., 1998).

II.1. Tachyzoites

This term was invented by Frenkel and refers to the rate or speed. Tachyzoite stage relates to the form of fast multiplication of *T. gondii* during the acute phase of infection. The tachyzoite is a crescent-shaped, 2-6 µm sized stage (Jones and Dubey, 2010). It includes various organelles such as mitochondria, microtubules, endoplasmic reticulum, ribosomes, Golgi complex ... The nucleus is typically located centrally in the cell (Figure 1).

Tachyzoites actively enter into the host cells through the plasma membrane. After penetration, the tachyzoite becomes oval and surrounded by a vacuole called parasitophorous vacuole. The invasion rates vary depending on the strain of *T. gondii* and the host cell type. After the entry of tachyzoite into a host cell, there may be a variable period of latency before the parasite divides (Dubey et al., 1998). Tachyzoites are sensitive to elevated temperature. They are killed by pasteurisation and heating. They died rapidly outside the host (Tenter et al., 2000).

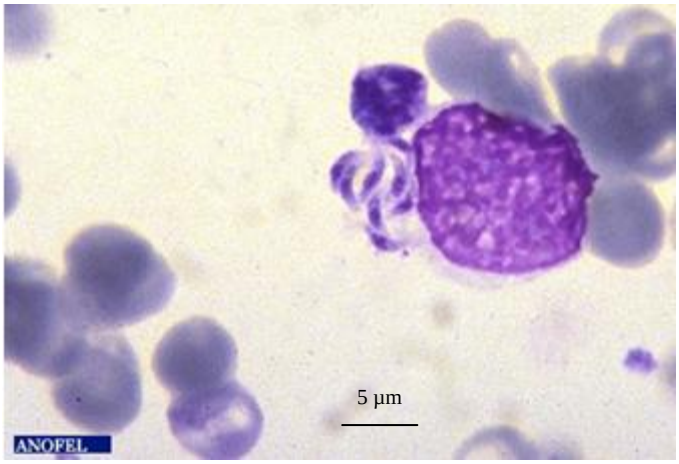


Figure 1: Tachyzoites (http://www.eanofel.fr).

II.2. Bradyzoites

The term bradyzoite also invented by Frenkel refers to a notion of slowness. This is the slow multiplication stage of *T. gondii* present during the chronic phase of infection. Bradyzoites are localized in tissue cysts. Cyst size is between 10 and 100 µM in diameter and the length of bradyzoites is between 5 and 6 µM (Figure 2). Cysts are located in various host cells but mainly in neurons and in heart and muscle cells (Dubey et al., 1998).

Bradyzoites are different from tachyzoites by the position of the nucleus, which is located rather to the posterior part. They contain multiple storage granules of amylopectin.

Cyst formation was observed from 6 days after infection in mice. These cysts may also rupture and release many bradyzoites, which will grow into tachyzoites that increases the power of the infection (Weiss and Kim, 2000).

The majority of tissue cysts appear as single structure in the host cell cytoplasm but it may be possible to find small groups of tissue cysts of different sizes. Tissue cysts of *T.gondii* are found in meat of any warm-blooded animal. They can persist for the life of the host (Tenter et al., 2000).

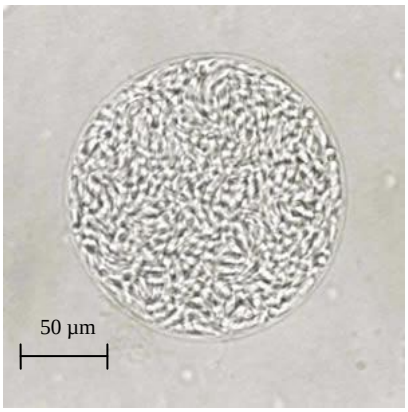


Figure 2: *Toxoplasma gondii* cyst (http://www.cdc.gov/toxoplasmosis/gallery/cystunstained).

II.3. Oocysts

Oocysts of *T.gondii* are the result of a sexual reproduction in cats or other felids (Figure 3). *Toxoplasma gondii* oocysts are excreted in the feces of domestic and wild cats. The oocyst stage is the stage of resistance in the external environment (Ferguson and Dubremetz, 2014). Upon excretion, oocysts in cat feces are unsporulated and non-infective. They mature in the environment, and, after sporulation, they contain 2 sporocysts with 4 sporozoites in each of them (Ferguson and Dubremetz, 2014). They are disseminated by rain and surface water, which leads to contamination of the environment. How often cats shed oocysts in the environment is unknown but cats can shed millions of oocysts after ingesting only one bradyzoite (Jones and Dubey, 2010). The sporulated oocysts of *T.gondii* are resistant to harsh climatic circumstances.

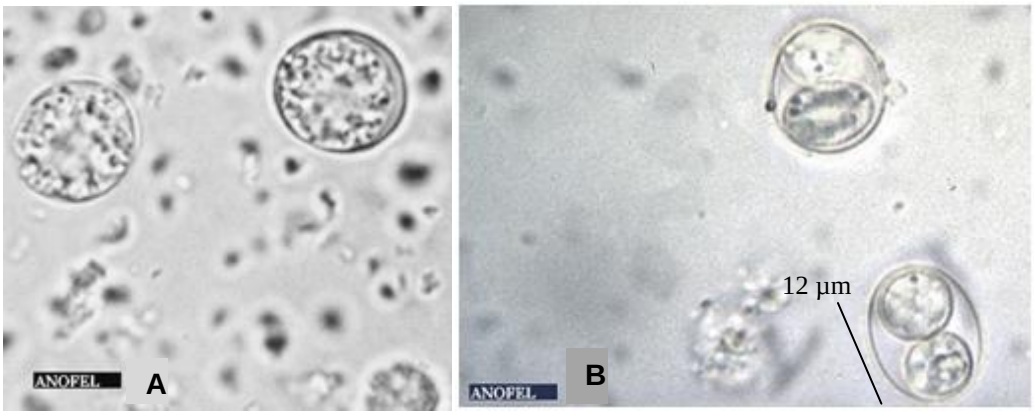


Figure 3: Unsporulated (A) and sporulated (B) oocyst of *T. gondii* containing 2 sporocysts (http://www.eanofel.fr).

III. Life cycle of *T.gondii*

The life cycle of *T.gondii* is complex. It comprises a phase of sexual reproduction in definitive hosts, which are the members of the Felidae family, especially cats. It also comprises a phase of asexual reproduction that occurs in intermediate hosts (birds and mammals) as well as in definitive hosts (Figure 4).

III.1. The asexual cycle

After ingestion of oocysts by the intermediate hosts (birds and mammals), the sporozoites release from the oocysts and penetrate into the intestinal epithelial cells, where they transform into tachyzoites. The tachyzoites are able to infect any nucleated cell type, and disseminate throughout the body via infected blood cells. After a few days, some of these tachyzoites transform into bradyzoites, gathered in cysts mainly found in nerve and muscle cells (Dubey, 2004). The cysts of *T. gondii* could persist throughout the host life. The mechanism of tissue cyst persistence is unknown. However, tissue cysts could break down periodically: bradyzoites transform to tachyzoites and reinvade host cells to form new tissue cysts (Tenter et al., 2000).

III.2. The sexual cycle

The sexual cycle occurs only in felines. After the ingestion by a cat of cysts present in tissues of an infected intermediate host, the parasites invade its intestinal epithelial cells. They firstly undergo a limited number of asexual multiplications (schizogony). Merozoites resulting from these first steps of asexual multiplications in enterocytes produce male or female gametocytes that will mature into gametes. The fertilization between male and female gametes leads to the production of unsporulated oocysts or non-infecting oocysts. These oocysts are excreted in the feces of Felidae and become sporulated in the environment, after a meiotic reduction leading to the production of sporozoites. The shedding of oocysts begins 4 to 6 days after the ingestion of tissue cysts and may continue up to 20 days. Cats can excrete several millions of oocysts that disseminate in the environment. The oocysts are very resistant and infective for both intermediate and definitive hosts (Dubey, 1998a; Innes, 2010).

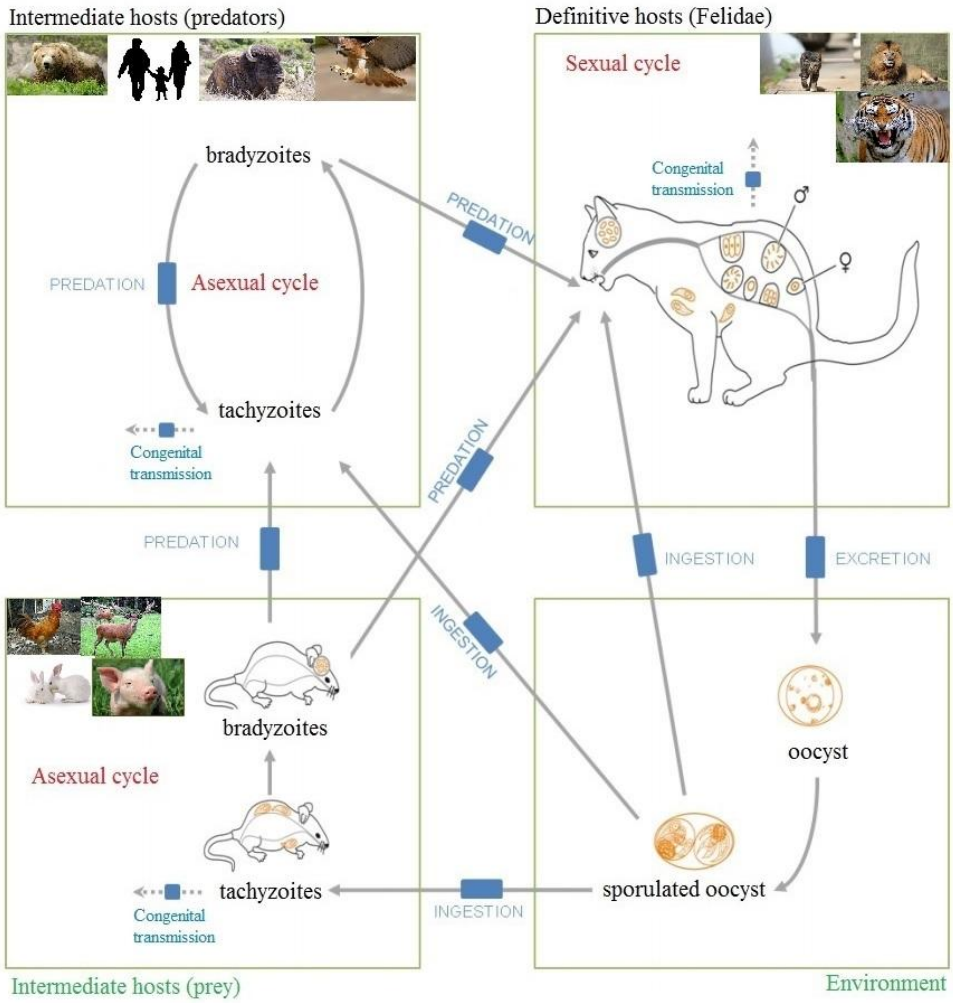


Figure 4: Life cycle of *Toxoplasma gondii* (modified from thesis of Dr. Aurélien Mercier, 2010).

IV. How do humans become infected?

The possible ways of contamination are:

1. Ingestion of tissue cysts by eating raw meat, undercooked or insufficiently frozen (lamb, pork, cow, beef, chicken, horse,...)

2. Ingestion of oocysts present in an environment contaminated by cat feces: plants (fruit, vegetable from the garden...), water, soil (gardening or farming activities), animal fur …

3. Direct contamination by cat by handling dropping litter in the absence of a proper hygiene.

5. Contamination through blood transfusion or organ transplant is quite possible although much infrequent (Robert-Gangneux and Dardé, 2012).

6. Transplacental transmission leading to a congenital infection of fetus when a woman acquires infection during pregnancy (Singh, 2016).

V. Human toxoplasmosis

V.1. Clinical features of toxoplasmosis

V.1.1. Acquired toxoplasmosis (postnatal, immunocompetent persons)

Infection by *T. gondii* is asymptomatic in over 80% of cases or causes a benign illness in approximately 20% of cases (Robert-Gangneux and Dardé, 2012). Symptomatic features classically associate fever, lymphadenopathy and asthenia. The patient will have a slight fever for a few days or weeks that will spontaneously disappear. The weakness can persist for several months. The evolution of the disease is usually benign and self-limited. Rarely, acquired infection in immunocompetent patients may lead to an ocular impairment with uveitis and retinochoroiditis. The percentage of patients with ocular impairment varies across continents, according to the circulating strains. The largest proportion of ocular lesions is observed in some parts of South America (Garweg, 2016). Exceptionally, severe forms of acquired toxoplasmosis can be observed in immunocompetent patients, with multivisceral failures. Lung involvement is predominant, leading to respiratory distress, and eventually to the death of these patients. These severe forms were mainly described in French Guiana due to the virulent strains circulating in the Amazonian rain forest (Carme et al., 2009).

V.1.2. Congenital toxoplasmosis

It derives from the contamination of the fetus during pregnancy. The most usual circumstance is the occurrence of a primary infection in pregnant women but the transmission may also occur in an immunocompromised pregnant woman by reactivation of tissue cysts

during a chronic infection. Congenital toxoplasmosis can lead to the abortion or to more or less severe symptoms according to the period of infection during pregnancy.

1. Severe congenital toxoplasmosis

Two clinical forms are distinguished. The first one is an encephalo-meningomyelitis, observed after a contamination in early pregnancy. It is responsible for microcephaly or macrocephaly with hydrocephalus, intracerebral calcifications and ocular involvement such as a pigmented retinochoroiditis. The second form is the form of serious neonatal infection (fever, jaundice, hepatosplenomegaly).

2. Benign congenital toxoplasmosis (degraded or delayed)

It corresponds to a late contamination during pregnancy and diagnosed at birth or during early childhood. The main symptoms are retinochoroiditis and/or intracerebral calcifications.

3. Latent congenital toxoplasmosis

It relates to newborns clinically normal at birth. Early treatment of these cases prevents their possible evolution to ocular or delayed neurological form (ANOFEL, 2014).

V.1.3. Toxoplasmosis in immunocompromised patients

This form is a serious illness, constantly threatening without treatment. Conventional descriptions distinguish localized forms and disseminated forms. In over 95% of cases, it is due to the reactivation of preexisting tissue cysts in immunocompromised persons (Luft and Remington, 1992). Reactivation occurs mainly in AIDS patients with less than 200 CD4 cells, but it can also occur in other types of immunodeficiency (solid organ transplanted patients, hematologic patients)

1. Localized toxoplasmosis

The most common location is the brain; the clinical characteristic is that of a cerebral abscess and encephalitis. Fever is present in 50% of cases and deficit focused in relation to the location of abscesses. The second most frequent location is eye. The patient complains of a declined visual acuity and eye redness. Pulmonary toxoplasmosis leads to a feverish interstitial pneumonia. The diagnosis methods used are pathological examinations, inoculation or molecular biology (Robert-Gangneux and Dardé, 2012).

2. Disseminated toxoplasmosis

Multiple organ impairment may be observed, involving CNS, heart, lungs and skeletal muscle (Cold et al., 2005).

V.1.4. Ocular toxoplasmosis

Most cases of ocular toxoplasmosis were classically thought to derive from a congenital infection, but it is now considered that the majority of cases are due to a postnatal infection. It is also observed as a localized form of reactivation in immunocompromised patients. Ocular involvement occurs in acute form such as blurred or altered vision. Infection area is the retina where cysts containing many quiescent bradyzoites are present. The rupture of these cysts causes the retina inflammation (Butler et al., 2013). A study using *in vivo* experimental models of ocular infection with *T. gondii* showed that the immunocompromised mouse model contained more parasite load than in immunocompetent mouse model. It indicated that the severity of the ocular disease increases due to the host immune system (Dukaczewska et al., 2015).

Chapter II: Southeast Asian countries and Toxoplasmosis

I. Description of Southeast Asian Region

Figure 5: Southeast Asian countries (http://www.nybg.org/southeast-asia-program).

Southeast Asian countries: currently**,** there are 11 countries in Southeast Asia including Brunei, Cambodia, East Timor, Indonesia, Laos, Malaysia, Myanmar, Philippines, Singapore, Thailand and Vietnam (Figure 5). These 11 countries are tropical countries, situated between the Indian Ocean 146°55'E and the Pacific Ocean 16°54'N, 100°0'W. Tropical climates

dominate the country with mountains and coastal areas covering the main land surfaces and may induce a high rate of seismic activity. There are around 639 million inhabitants in Southeast Asia (Table 2). The ethnic groups of Southeast Asia are a result of the emergence of local differences among people who evolved into identifiable cultural or ethnic groups. Many distinct groups can exist and each group has its own language and tradition. Nowadays, the globalization process increases the interaction and communication opportunities between groups. Economic activities vary according to the country. Singapore is an economic tiger. Brunei is an oil-rich production. Thailand is a major manufacturing center. Philippines is a destination for outsourced information jobs. Laos and Myanmar have weak economies. Vietnam and Cambodia are recovering from political isolation. The majority of population in these countries is Buddhist and the three main religions found in these countries are Buddhism, Catholic and Islam.

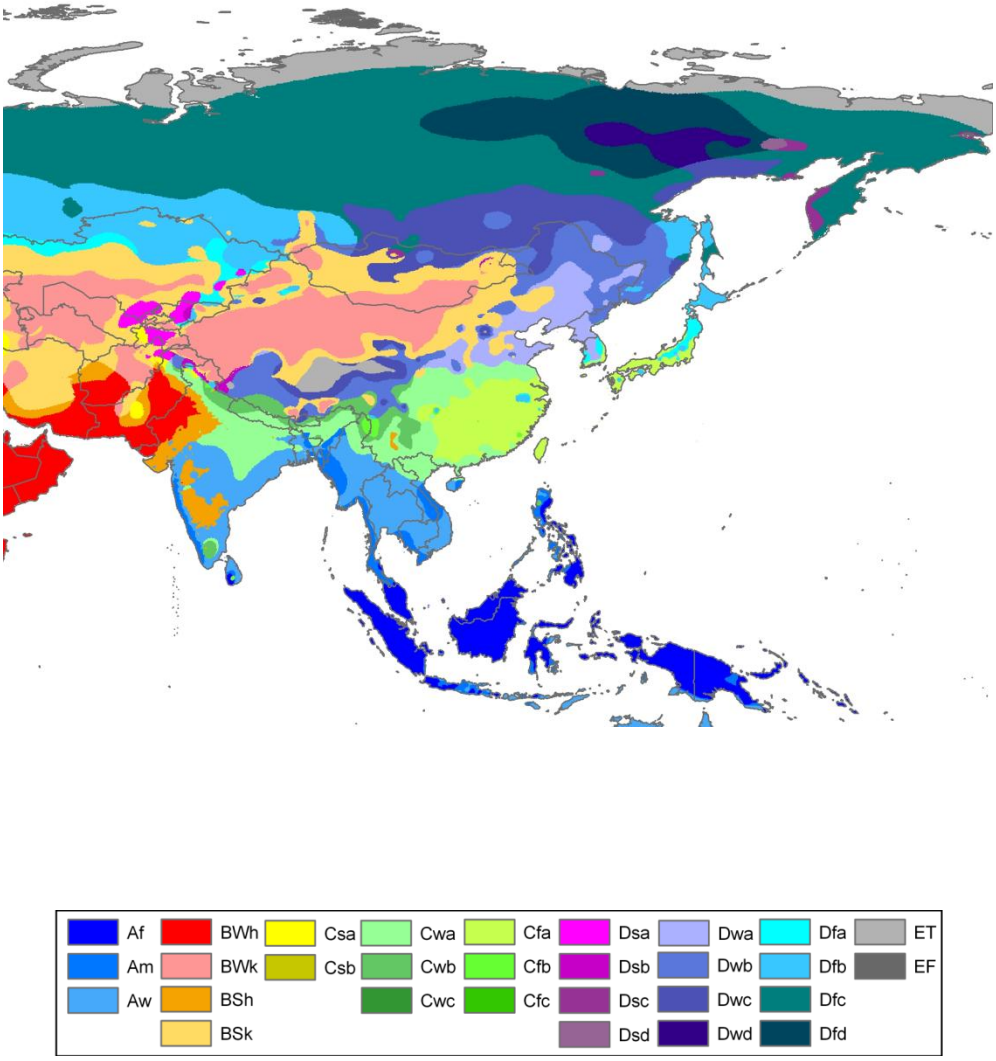
Table 2: General information on Southeast Asian Countries.

Countries	**Inhabitants ($x10^6$)**	**Religion**	**Capital**
Brunei	0.4	Islam	Bandar Seri Begawan
Cambodia	15.8	Buddhism	Phnom Penh
East Timor	1.2	Roman Catholicism	Dili
Indonesia	260.0	Islam	Jakarta
Laos	6.9	Buddhism	Vientiane
Malaysia	30.7	Islam	Kuala Lumpur
Myanmar	54.3	Buddhism	Naypyidaw
Philippines	102.2	Roman Catholicism	Manila
Singapore	5.6	Buddhism, Islam, Christianism	Singapore
Thailand	68.1	Buddhism	Bangkok
Vietnam	94.4	Buddhism	Hanoi
Total	**639.6**		

Source: http://www.geohive.com

II. Climatic characteristics of Southeast Asia

The dark blue areas show that the section is of equatorial climate in other words it is a tropical/tropical rainforest climate. The darker light blue is monsoon climate (Figure 6). Equatorial region comprises Indonesia, Malaysia, East Timor, Brunei and Singapore. Characteristic conditions of this equatorial zone are the well-distributed rainfall and high temperature for the whole year. Thailand, Vietnam, Laos, Myanmar, Philippines and Cambodia are part of tropical monsoon region. The typical condition of tropical monsoon region is the monsoon circulation between ocean and land. The annual rainfall and average temperature are quite similar to equatorial zone (http://geographynote.com).



Af: equatorial climate	**Dsa**: warm continental climate/mediterranean continental climate
Am: monsoon climate	**Dsb**: temperate continental climate/mediterranean continental climate
Aw: tropical savanna climate	**Dsc**: cool continental climate
BWh: warm desert climate	**Dsd**: cold continental climate
BWk: cold desert climate	**Dwa**: warm continental climate/humid continental climate
BSh: warm semi-arid climate	**Dwb**: temperate continental climate/humid continental climate
BSk: cold semi-arid climate	**Dwc**: cool continental climate/subarctic climate
Csa: warm mediterranean climate	**Dwd**: cold continental climate/subarctic climate
Csb: temperate mediterranean climate	**Dfa**: warm continental climate/humid continental climate
Cwa: humid subtropical climate	**Dfb**: temperate continental climate/humid continental climate
Cwb: humid subtropical climate/subtropical oceanic highland climate	**Dfc**: cool continental climate/subarctic climate
Cwc: oceanic subpolar climate	**Dfd**: cold continental climate/subarctic climate
Cfa: warm oceanic climate/humid subtropical climate	**ET**: tundra climate
Cfb: temperate oceanic climate	**EF** : ice cap climate
Cfc: cool oceanic climate	

Figure 6: Southeast Asian climate (http://www.wikimedia.org).

III. Focus on Thailand and overview (our study area)

As our study was conducted in Thailand, whereby we gave more details concerning Thailand than other Southeast Asian countries. Thailand is located in Southeast Asia, occupies the western half of the Indochinese peninsula and the northern two-thirds of the Malay Peninsula in Southeast Asia. The official language is Thai. It is bordered to the north by Myanmar and Laos, to the south by Malaysia, to the east by Cambodia and Laos and to the west by Myanmar. It represents a total area of approximately 513,000 km^2. The capital of Thailand is Bangkok, the most anthropized province of Thailand.

III.1. Geography

Thailand geography is also characteristic variety of landscape. It includes central plain, mountains scattered and plateau. The north is mountainous along the boundary with Myanmar on the west (Figure 7). The environment of Thailand faces threat from air pollution, water pollution, soil erosion, deforestation and illegal hunting of wild life, which destroy the ecological balance.

Figure 7: Thailand map (http://www.freeworldmaps.net/asia/thailand).

III.2. Climate

Thailand is situated in the tropical geographical zone 15° 00' North latitude and 100° 00' East **longitude** and has a tropical climate. The climate varies in different parts of the country. In general, it comprises 3 climatic seasons, hot season, cold season and rainy season but predominantly hot and humid. The weather in Thailand shows distinct variations between the northern part (dry and cold) of the country and the southern areas (hot and humid). The temperature in Thailand averages from 18 degrees to 40 degrees Celsius. The temperatures are generally cooler in the highlands especially mountains and forest zones and along the coast. The temperatures can sometimes drop to near zero at night in the higher elevations of northern Thailand in the cold season. The southern is always hot and humid.

Hot and humid climate due to tropical geographical zone might induce the tropical cyclones such as depressions, tropical storms and typhoons with the serious consequence for the country such as the flood.

Seasonality: Thailand has very low seasonality. Its temperatures remain fairly constant (hot and humid), and precipitation, with the exception of a few of the winter months, is heavy (Figure 8).

Bangkok and Phuket are 2 examples that show this:

In Bangkok, the average of yearly precipitation is 1,467 mm and the average of yearly temperature is 28.1 °C.

In Phuket, the average of yearly precipitation is 2,383 mm and the average of yearly temperature is 27.6 °C.

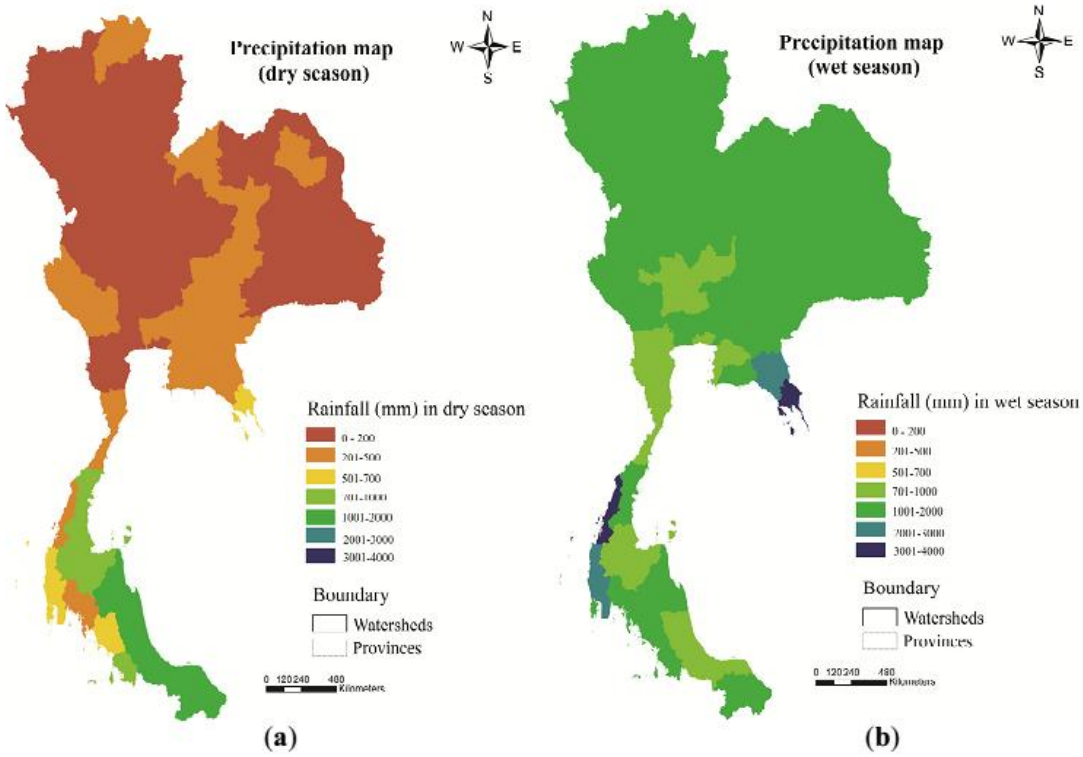


Figure 8: Precipitation maps of Thailand (a) Dry season; (b) Wet season (Gheewala et al., 2014).

The average of annual rainfall in Thailand is about 1,300 mm but some part of the country, especially in Northeast region, which continue to have the drought problem. The drought problem becomes more serious in summer than in other seasons (Figure 9).

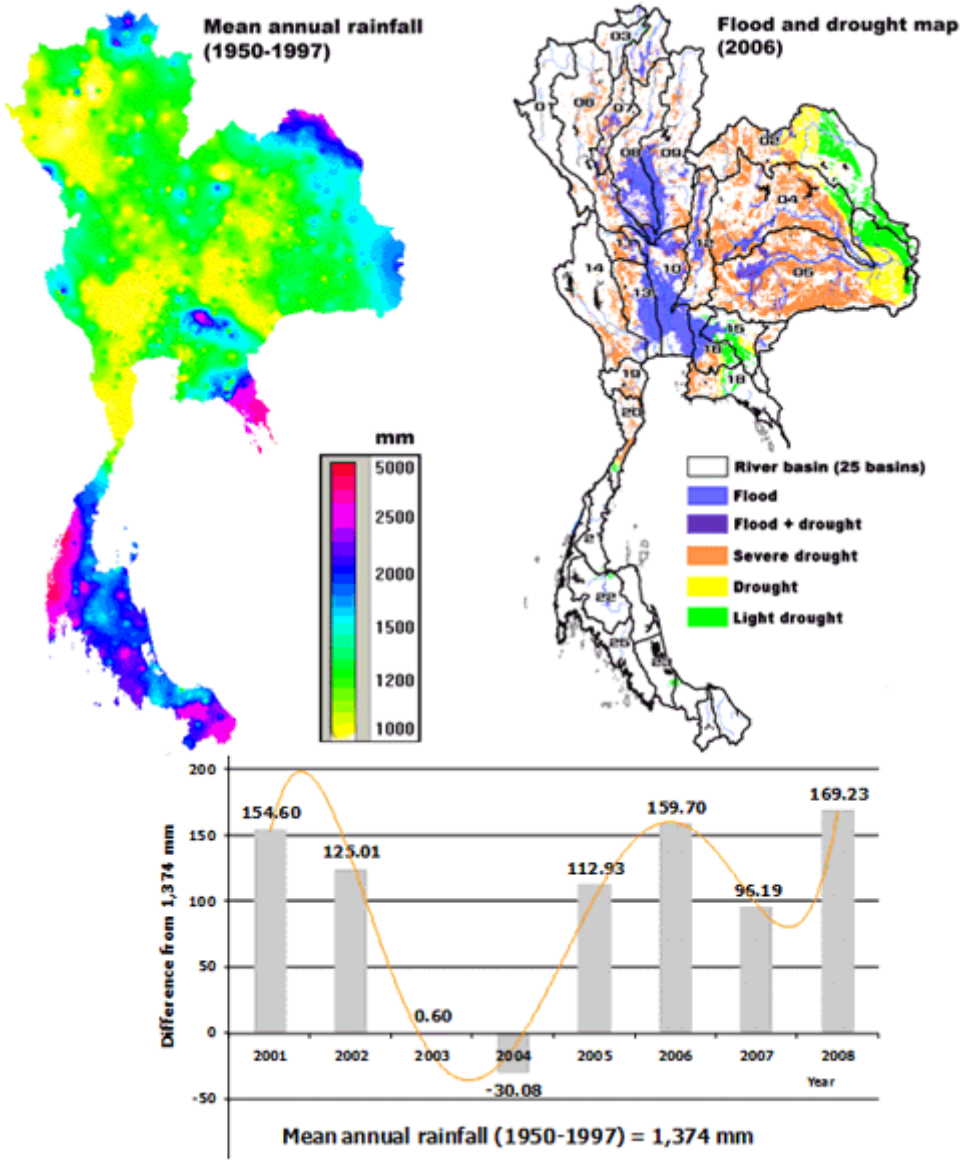


Figure 9: The distribution of annual rainfall average and the drought map (http://www.thaiwater.net).

III.3. Human population

III.3.1. Socio-economic levels

The social and economic development of Thailand remarkably progresses and moves from a low-income country to an upper-income country in a few time. The economy of Thailand grows at the annual average of about 7.5%. Currently, there are more children who are getting more years of education. The health insurance becomes necessary for everyone. The poverty has declined over the last 30 years (http://www.worldbank.org). The provinces in green color, which represents the high level of population incomes, are mainly the industrial provinces. The majority of populations who live in the provinces (dark red) are farmers. Their incomes are quite low when compared with other provinces (Figure 10).

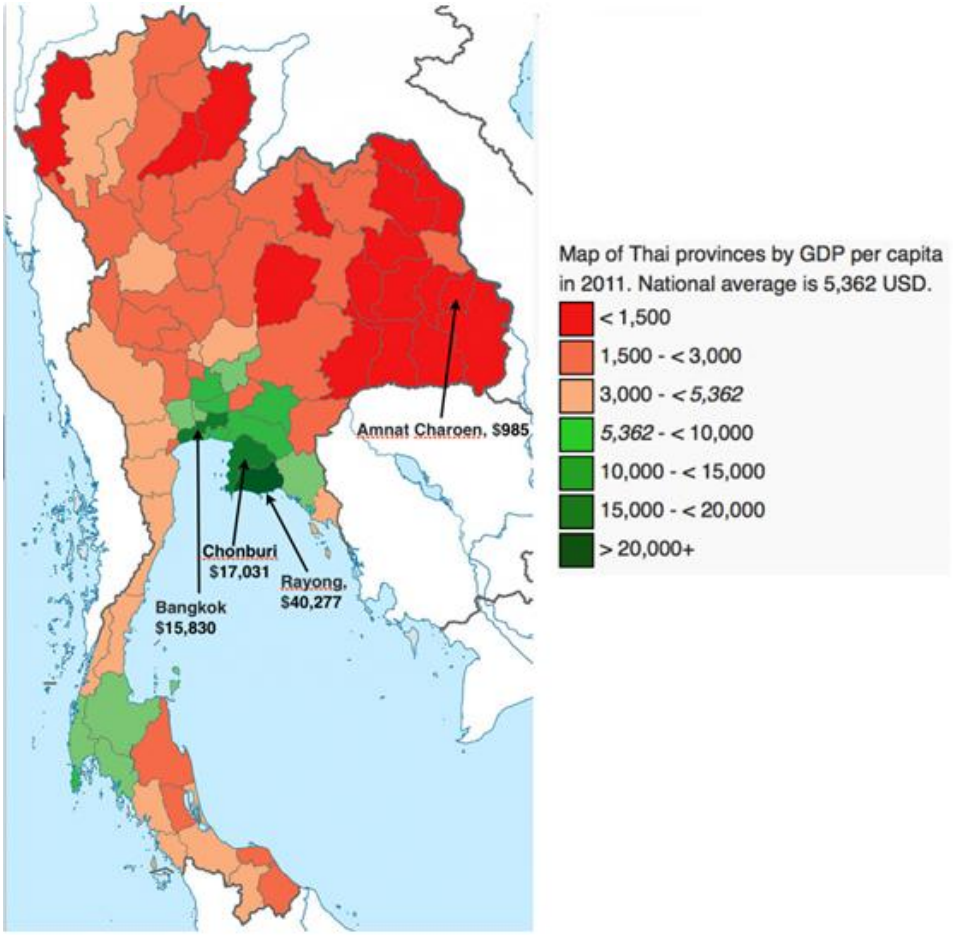


Figure 10: Thai average incomes classified by provinces (http://www.wikipedia.org/wiki/List_of_Thai_provinces_by-GPP).

III.3.2. Traditional Thai culture

The basis of Thai traditions lies in the family like the Chinese and some other Asian people, the young are taught to pay respect and follow the parents, elders, teachers and Buddhist monks. About traditional Thai culture, what cannot be left unmentioned is the Buddhist temple. Buddhism religion had been spread throughout Thailand for hundreds of years. Thai population was assimilated by the Buddhist and the temple became the centre of the village. Nowadays, due to the rapid advancement of technology, tradition Thai culture has inevitably changed mostly in the big cities. However, it is still preserved in the faraway rural areas whereas the modern civilization has failed to penetrate.

In Thailand, there are several ethnic groups mostly living at the border of the country such as the Khmer, at the border to Cambodia. The Malays at the border to Malaysia and the Karen near the frontier of Myanmar (Figure 11). Most of these ethnic groups do not have the same socio economic conditions than Thai people. The majority of them are farmers, they live near the forests, mountains and rivers. Their houses are poorly constructed and in some cases, there are no toilets in their houses. Lack of good hygiene and cleanliness can be found in most of them (http://www.thaimissions.info).

Life style, occupation and life conditions of ethnic groups in Thailand may represent risk factors for *T. gondii* infection. Unfortunately, there are no seroprevalence studies in these ethnic groups due to the difficult accessibility in their villages (Figure 12) and to the communication problems: some of them cannot speak Thai language. It would be interesting to carry out the seroprevalence studies in ethnic groups in order to compare with general Thai population.

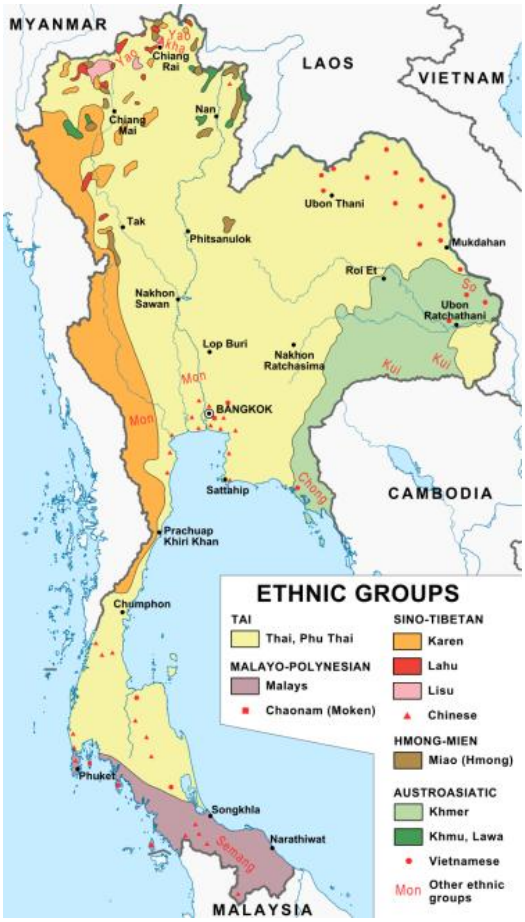


Figure 11: Different ethnic groups in Thailand (https://en.mwikipedia.org/Ethnic_groups/Thailand).

Figure 12: Accommodation of ethnic groups in Thailand (http://www.thaimissions.info).

III.3.3. Regional Thai kitchen

The cooking preparation is different in each Thai region, northern, central, northeastern and southern regions.

Rice is strictly the staple food for every family in the central region. Thai meals of the central region are vegetable, namprik (chilli sauce), platoo (type of fish) omelette (Thai style) and Thai beef or roasted pork well cooked. Thai food in southern region is spicier than in other regions of Thailand. Favored dished are a whole variety of spiced soup with well cooked meat. The northern people have penchant for medium cooked food with a touch of salty tastes almost to the exclusion of sweet and sour tastes. Meat preferred by the northern people is pork followed by beef, chicken, duck, bird and other. Sea food is the least known on account of the remoteness of the northern region from the sea. The glutinous rice or sticky rice is served for every meal.

Same as Thai food in the north, Thai food of the northeast has steamed glutinous rice as a staple base to be taken with spiced ground meat with red pork blood. The northeastern populations prefer to have their meat fried and the meat could be frog, lizard, snake, rice field rat, large red ants and insects. Pork, beef and chicken are preferred according to different families.

Therefore, in northern and northeastern regions, the population might have a higher probability to be infected by *T. gondii* due to their cooking preparation especially in northeast when people eat their traditional dish; Koy is a dish cooking with raw meat (pork, beef and fish).

Conclusion: Thailand presents a large diversity regarding aspects that could influence *Toxoplasma* epidemiology: diversity of climatic conditions (rainfall index, risk of flood), diversity in land occupation (highly urbanized areas, rural areas dedicated to rice cultivation or other, bush areas, remains of tropical rainforest), and diversity of population (socio-economic levels; ethnic groups; culinary habits; lifestyle). Epidemiological studies (seroprevalence, molecular epidemiology) taking into account these diverse conditions would be interesting to better analyze their role as risk factors for *Toxoplasma* infection in Thailand. For instance, what is the influence of periodic floods in the Bangkok area: dispersion of oocysts, reduction of environmental contamination? What will be the influence of the rapid climatic and landscape changes occurring in this country? Is the seroprevalence rate higher in ethnic populations due to their lower socio-economic status? What is the influence of urbanization on *Toxoplasma* epidemiology?

IV. Literature review

IV.1. Seroepidemiology of *Toxoplasma* infection in Southeast Asian countries

Literature review on *Toxoplasma* infection seroprevalence in Southeast Asian countries was performed from July 2016 to August 2016 with the following databases, *Medline and Google scholar*. The keywords and the following research equations were used. The literature review was performed only in English (Figure 13).

Toxoplasma [OR] Toxoplasmosis [AND] prevalence [AND] Southeast Asia

Toxoplasma [OR] Toxoplasmosis [AND] prevalence [AND] each country in Southeast Asia including Brunei, Cambodia, East Timor, Indonesia, Laos, Malaysia, Myanmar, Philippines, Singapore, Thailand and Vietnam

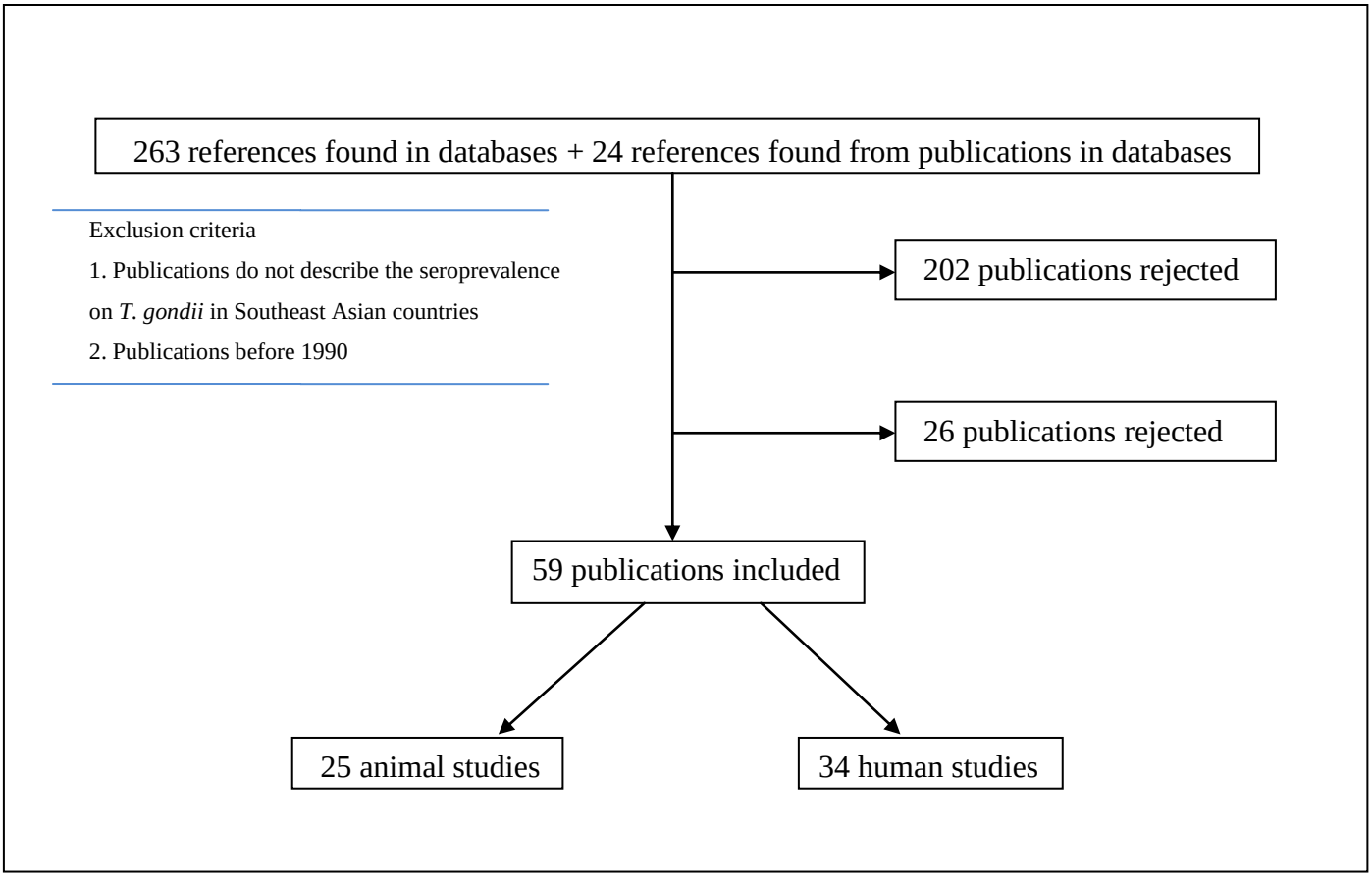


Figure 13: Literature search showing selection procedure for the publications.

The publications before 1990 were excluded because our literature review study focused on the current toxoplasma seroprevalence. The information from these 59 publications was used to make the seroprevalence tables. There are few studies in Southeast Asian countries in comparison to other continents. All seroprevalence studies including all animal species and all type of studied population groups were included in this literature review study. The majority of publications concerned Malaysia and Thailand. Confidence intervals 95% CIs of seroprevalence rates derived from the relevant study. When CIs were not available in the publications, the estimation of 95% CIs was done using Microsoft Office Excel Software with the statistical formula used below.

$$\bar{x} \pm 1.96\left(\frac{\sigma}{\sqrt{n}}\right)$$

$\bar{x}$ = average

σ = standard deviation

n = total number of individuals in the sample

IV.2. Seroprevalence of *T. gondii* in animals

The overall seroprevalence of toxoplasmosis found in different animal species in Southeast Asian countries ranged from 3.0% to 100.0% (Table 4). There was a disparity of seroprevalence rates found among different animals and in different parts of the country. The disparity may be due to the different serological techniques used as well as to the risk factors, which might cause *T. gondii* infection.

IV.2.1. Seroprevalence in intermediate hosts

The intermediate hosts may be infected by the ingestion of cysts or oocysts of *T. gondii*. All mammal species and birds could play a role as intermediate hosts or reservoir of *T. gondii*. The carnivores can be contaminated by the consumption of infected meat contrary to the herbivorous, which can be infected by the consumption of contaminated vegetables. The omnivorous can be infected by both contamination sources.

1. Seroprevalence in the animal species commonly used for consumption by humans

1.1. Free range chickens

The seroprevalence rate of free-range chickens ranged from 24.4% in Indonesia (Dubey et al., 2008) to 64.03% in Thailand (Chumpolbanchorn et al., 2009). This difference in seroprevalence could be due to different serological techniques used, MAT and IFAT, and to environmental risk factors. The high prevalence found in Thailand was observed in a serological study of free-range chickens in Bangkok using IFAT (Chumpolbanchorn et al.,

2009). Although chickens are considered as an important source of *T. gondii* infection, people generally consume chicken meat well cooked except in cases of street foods such as the skewers of grilled chicken.

1.2. Pigs

The seroprevalence found in pigs ranged from 6.3% in Indonesia (Inoue et al., 2001) to 71.43% in Thailand (Thiptara et al., 2006). A study in Peninsular Malaysia showed no infected pig, this may result from the good farm management (Chandrawathani et al., 2008). In Thailand, the study was carried out in a small farm and a cat was found positive in the farm, this positive cat may explain the high seroprevalence in pigs (Thiptara et al., 2006). Pork is widely consumed in the population but the majority of population in Brunei, Indonesia and Malaysia is Muslim. According to Islam religion, they do not eat pork.

1.3. Goats

High seroprevalence in goats was found in Indonesia with 47.5% (Matsuo and Husin, 1996) followed by 35.5% in Malaysia (Chandrawathani et al., 2008), 27.9% in Thailand (Jittapalapong et al., 2005) and 11.4% in Myanmar (Bawm et al., 2016). A traditional dish of Indonesian people is Sate, a dish with grilled goat or chicken. As this meat is generally insufficiently cooked, this may be a source of infection in Indonesian population (Matsuo and Husin, 1996).

1.4. Cattle

The seroprevalence rate found in cattle was quite low. It ranged from 3.8% in Malaysia (Normaznah et al., 2004) to 25.7% in Thailand (Wiengcharoen et al., 2012). In a study on dairy cows in Thailand, the seroprevalence was 9.4% by LAT and 17.0% by ELISA showing that seroprevalence rates may largely be influenced by the technique used for detecting antibodies (Inpankaew et al., 2010). In the majority of farms, cats have access to water and food of the livestock and excrete around the farm.

1.5. Free-range ducks

A study showed 14.6% of *T. gondii* seroprevalence in Malaysia (Puvanesuaran et al., 2013b). Malaysia is an important duck meat producer and there is a wide free-range duck market. Pregnant women are encouraged to consume free-range meat because the commercially bred meat is tainted with growth hormones and antibiotics that might damage

the fetuses. Free-range duck meat might be a source of *T. gondii* contamination in pregnant women in Malaysia (Puvanesuaran et al., 2013b).

2. Seroprevalence in wild species and rarely consumed animal species

2.1. Rodents

A low seroprevalence was found in rodents in Thailand and Malaysia, 4.6% (Jittapalapong et al., 2011) and 5.9% (Normaznah et al., 2015) respectively but high seroprevalence was found in rats in Philippines with 55.0%. Rodents survive and proliferate around human habitation and they are considered important pests for the agricultural damages. They can play a role as a parasite reservoir to cats (Salibay and Claveria, 2005).

2.2. Wild boars

In the forests of Pahang, Malaysia, 100% seroprevalence was found in 30 wild boars. Wild boar meat is considered a delicacy in Malaysia and often enjoyed by the ethnic Chinese community (Puvanesuaran et al., 2013a). People who live in rural areas especially near the forests are used to eat wild animals including wild boars, potential source of contamination.

3. Seroprevalence in domestic animals

3.1. Dogs (domestic and stray dogs)

The seroprevalence in dogs was quite low, 9.6% in Malaysia (Chandrawathani et al., 2008), 9.4% (Jittapalapong et al., 2007) and 10.9% in Thailand (Jittapalapong et al., 2009). Dogs are normally domestic animals except in some provinces of Laos, Thailand and Vietnam where some people eat dog meat. In Thailand, there are many street dogs, living mainly in monastery places and in the parks. The fur of dogs is a major source of *T. gondii* transmission because dogs like to seek and roll in cat feces that lead to contaminate their fur with oocysts of *T. gondii*. Children like to play with dogs and touch their fur, and some of them put the fingers in their mouth. This habit is considered as important risk factor for *T. gondii* contamination (Frenkel et al., 2003).

IV.2.2. Seroprevalence in definitive hosts

Felidae particularly wild and domestic cats are the source of *T. gondii* environmental contamination. The definitive hosts of *T.gondii* are cats and other felines. They are considered the main reservoir of infection and play the important role in the spread of *T. gondii* infection because they are the only animals that excrete resistant oocysts in the environment. They are

contaminated by eating meat from infected animals or by the ingestion of oocysts contaminating the environment and vegetables. For indoor-only cats, the most likely source of infection is uncooked meat scraps or raw meat. A cat may release in transmission period (7-15 days) up to 10 million oocysts per day.

1. Wild felids (Annex 1)

It remains currently 10 species of wild felids in Southeast Asia as shown in Table 3. *Panthera tigris* (Tiger), *Neofelis nebulosa* (Clouded leopard), *Catopuma temmincki* (Asiatic golden cat), *Prionailurus planiceps* (Flat-headed cat), *Prionailurus viverrinus* (Fishing cat), *Pardofelis marmorata* (Marbled cat) *and Panthera pardus (Leopard)* are endangered species with a high extinction risk, while *Catopuma badia* (Bornean bay cat), *Felis chaus* (Jungle cat) and *Prionailurus bengalensis* (Leopard cat) are widespread and abundant species (Macdonald et al., 2010).

Two seroprevalence studies were performed on captive wild felids from different regions of Thailand. Seroprevalence ranged from 15.4% from 136 captive felids of 12 species (Thiangtum et al., 2006) to 42.8% from 21 captive wild felids of 8 species (Buddhirongawatr et al., 2006). The highest prevalence (42.8%) was found in wild felids in an open zoo where they may be in contacts with other animals. Three species among them, Leopard cat (*Felis bengalensis*), Cheetah (*Acinonyx jubatus*) and Lion (*Panthera leo*), showed the ability to shed oocysts in the environment (Jones and Dubey, 2010). The disparity of seroprevalence may also be explained by the use of different serological techniques used, LAT and Sabin Feldman Dye Test respectively. These seroprevalence studies in captive animals do not allow evaluating the role of these wild felids in the maintenance of *T. gondii* cycle in wild areas, but we did not find any study on prevalence in free-living wild felids in South-East Asia.

2. Cats (Annex 1)

The seroprevalence rate ranged from 4.8% in Thailand (Jittapalapong et al., 2010) to 14.5% in Malaysia (Chandrawathani et al., 2008). It was quite low. Most cats are raised either outdoors or both indoors and outdoors. In Thailand, cats are kept as pets but they can go freely outdoors. Cats are one of the most popular domestic animals and are frequently in close contact with humans. In Thailand, the majority of stray cats were found mostly in the monasteries. They should excrete around the temples and the oocysts can be spread by the rain (Sukthana, 2006). Most of cats in Thailand live outdoors including pet cats and stray cats. They are fed by rice and well cooked fish. This differs from cats in European countries, which are fed by raw/undercooked meat, and may be an explanation for the low prevalence of

toxoplasma infection in this country (Sukthana, 2006). Cats and dogs are common domestic animals in Southeast Asia.

Table 3: Felidae distribution in Southeast Asian countries.

Animals	Name of species	Distribution
Tiger	*Panthera tigris*	Cambodia, Indonesia, Laos, Malaysia, Myanmar and Thailand
Bornean bay cat	*Catopuma badia*	Brunei, Indonesia, Malaysia
Clouded leopard	*Neofelis nebulosa*	Indonesia, Laos, Malaysia, Myanmar, Thailand and Vietnam
Asiatic golden cat	*Catopuma temmincki*	Cambodia, Indonesia, Laos, Malaysia, Myanmar, Thailand and Vietnam
Flat-headed cat	*Prionailurus planiceps*	Brunei, Indonesia, Malaysia and Thailand
Fishing cat	*Prionailurus viverrinus*	Cambodia and Thailand
Marbled cat	*Pardofelis marmorata*	Cambodia, Indonesia, Laos, Malaysia, Myanmar, Thailand and Vietnam
Leopard	*Panthera pardus*	Cambodia, Indonesia, Laos, Malaysia, Myanmar, Thailand and Vietnam
Jungle cat	*Felis chaus*	Cambodia, Laos, Myanmar, Thailand and Vietnam
Leopard cat	*Prionailurus bengalensis*	Brunei, Cambodia, Indonesia, Laos, Malaysia, Myanmar, Philippines, Singapore, Thailand and Vietnam

Conclusion: The seroprevalence of *T. gondii* infection was quite high in the common consumption animals such as free-range chickens, pigs and goats. These seroprevalence rates are not fundamentally different from those observed in European countries (Dubey, 2010a). The human population could be contaminated when consuming these meats not adequately cooked. For the wild species, the most concerned people are those who live in the forests such as the ethnic groups and the hunters. There is now a large population of domestic and stray cats, source of environmental contamination (water, soil, plants, animal furs) in many urban and rural areas of Southeast Asia. This could lead to an increase in *Toxoplasma* circulation in animals as well as in humans.

Toxoplasma seroprevalence studies in wild species in Southeast Asia, difficult to undertake, especially in remote areas, are lacking to better understanding the transmission of the parasite in this environment and the role of wild felids. However, the high rate of prevalence found in the only study performed in wild animals (wild boars from Malaysia forest) indicates the existence of a sylvatic cycle of *T. gondii*, probably maintained by wild felids.

Due to the diversity of animal species, and of animal sampling presented in these studies, it is difficult to conclude to differences among the Southeast Asian countries regarding circulation of *T. gondii* in the environment.

More studies will be needed in the various animal species and in different environments (wild, rural or urban areas) in order to provide a better knowledge concerning the *Toxoplasma* epidemiology through Southeast Asian countries.

Table 4: *Toxoplasma gondii* seroprevalence in animals in Southeast Asian countries.

Countries	Samples	Sample size	Prevalence % (95% confidence interval)	Technique	References	Comments
Indonesia						
12 towns	Free-range chickens	98	24.4 (15.9-32.9)	MAT	Dubey et al., 2008	
Lumpung	Goats Cattle	160 200	47.5 (39.8-55.2) 9.0 (5.0-13.0)	LAT (Toxocheck-MT, Eiken)	Matsuo and Husin, 1996	Goats play a more important role than cattle in *T. gondii* infection Sate is a traditional Indonesian dish with grilled goat meat. The preparation of this food was not clean and the cooking of goat meat was not adequate
Western Java	Cattle Pigs	598 205	7.4 (5.4-9.8) 14.6 (10.1-20.2)	ELISA TgGRA7 antigen	Ichikawa-Seki et al., 2015	Higher seroprevalence in pigs than in cattle
2 slaughter houses, Bandar Lampung, Sumatra and Ujung Pandang Sulawesi	Pigs	208	6.3 (3.0-9.6)	LAT (Toxocheck-MT, Eiken)	Inoue et al., 2001	Higher seroprevalence rate in Ujung Pandang than in Bandar Lampung
Malaysia						
Rural and forest areas in Peninsular	Rodents	526	5.9 (3.9-7.9)	IFAT	Normaznah et al., 2015	The rat species with the highest prevalence was *Rattus argentiventer*
4 states in Peninsular, Kedah and Perak in the northern region Melaka and Johor in the southern region	Free-range ducks	205	14.6 (9.8-19.4)	MAT	Puvanesuaran et al., 2013b	High specificity of detection when MAT titers was 1:24
Peninsular, forests in Pahang	Wild boars	30	100.0 (100.0-100.0)	MAT	Puvanesuaran et al., 2013a	Wild boar meat is considered a delicacy in Malaysia and often enjoyed by the ethnic Chinese community
Peninsular	Pigs	100	0.0 (0.0)	IFAT	Chandrawathani et al., 2008	No antibodies were detected to *T. gondiii* n pig serum samples. This may be due to good farm management
	Goats	200	35.5 (28.9-42.1)			
	Cattle	126	6.3 (2.1-10.5)			
	Dogs	135	9.6 (4.6-14.6)			
	Cats	55	14.5 (5.2-23.8)			

Gombak District, Selangor	Cattle	73	3.8 (0.6-8.2)	IFAT	Normaznah et al. , 2004	Low prevalence in cattle may suggest the low risk of acquiring the infection from consuming undercooked beef
Myanmar						
8 farms in 3 cities, Nay Pyi Taw, Mandalay and PyinOoLwin	Domestic goats	119	11.4 (5.7-17.1)	LAT (Toxocheck-MT, Eiken)	Bawm et al., 2016	Presence of cats in the farms, farming with different animal species, and farming without good management practices presented association with the infection
Philippines						
Dasmariñas	Rats	157	55.0 (47.2-62.8)	LAT (TOXOCELL AD Direct Agglutination Test Kit)	Salibay and Claveria, 2005	Statistical analysis showed no significant association between parasite infectivity, rat species (*Rattus norvegicus* and *Rattus rattus mindanensis*) and collection sites
Thailand						
Bangkok suburbs	Free range chickens	303	64.03 (58.6-69.4)	IFAT	Chumpolbanchorn et al., 2009	Chickens were from small farms with no farm-biosecurity and cats were often observed. All chickens were older than 5 months.
Kanchanaburi, Ratchaburi and Nakhon Pathom	Cattle	389	25.7 (21.4-30.0)	IFAT	Wiengcharoen et al., 2012	The animals were of both sexes 1-6 years old Cats and dogs have access to the water and food of the livestock and excrete around the farm
13 provinces, rural areas	Rodents	461	4.6 (2.7-6.5)	LAT (Toxocheck, Eiken)	Jittapalapong et al., 2011	18 rodent's species included

						Very low seroprevalence
Bangkok	Stray cats	1490	4.8 (3.7-5.9)	Sabin Feldman Dye Test	Jittapalapong et al., 2010	All 50 districts of Bangkok were sampled. 15 to 30 cats form each district. Fifty-six percent (28/50) of the districts examined had *T. gondii* positive cats
Chiang Mai, Chiang Rai, Lumpang and Mae Hong Son, northern	Cows	700	9.4 (7.2-11.6) 17.0 (14.2-19.8)	LAT (Toxocheck Eiken), Elisa (in house)	Inpankaew et al., 2010	In most dairy farms in Thailand, cats are frequently found in the farm's perimeter and their feces might contaminate farm areas
Bangkok, a district in center of Bangkok	Stray dogs	230	10.9 (6.9-14.9)	LAT (Toxocheck, Eiken)	Jittapalapong et al., 2009	Significantly higher seroprevalence in males than in females
Chonburi, Khao Kheow Open Zoo	Captive wild felids	21	42.8 (21.6-64.0)	Sabin Feldman Dye Test	Buddhirongawatr et al., 2006	Clouded leopard and tiger were the 2 species mostly infected
Monasteries in Bangkok	Stray cats	427	11.0 (8.0-14.0)	LAT (Toxocheck, Eiken)	Jittapalapong et al., 2007	Most cats are raised either outdoors or both outdoors and indoors No significant difference was observed between the sexes
	Stray dogs	592	9.4 (7.0-11.8)			
Nakhonsithammarat, small holding farm Southern	Piglets	14	71.43 (47.8-95.1)	LAT (Toxocheck, Eiken)	Thiptara et al., 2006	A cat in this farm was positive
Different regions	Captive wild felids	136	15.4 (9.3-21.5)	LAT (Toxocheck, Eiken)	Thiangtum et al., 2006	Samples were collected from 136 captive felids of 12 species

Satoon province, Southern	Domestic goats	631	27.9 (24.4-31.4)	LAT (Toxocheck, Eiken)	Jittapalapong et al., 2005	Significantly higher seroprevalence in female goats than in male goats Goats more than 1-year-old showed higher seroprevalence than other age groups
Bangkok	Cats	315	7.3 (4.4-10.2)	Sabin Feldman Dye Test	Sukthana et al., 2003	In Thailand, cats are kept as pets but they are allowed to roam freely outdoors Close association with infected cats was a risk for acquiring Toxoplasma infection in human adults
Vietnam						
Rural areas, Dong Nai, Ben Tre, Tien Giang, Long An, Tra` Vinh, and Vinh Long, 38 properties	Free range chickens	330	24.2 (19.6-28.8)	MAT	Dubey et al., 2008	
Different parts of the country	Cattle	200	10.5 (6.3-14.7)	Direct agglutination test DAT; Toxo-screen, BioMerieux	Huong et al., 1998	The cattle were 2–6-year-old 170 were from private farms keeping 5 to 10 dairy cows, and 30 from a state farm
	Water buffaloes	200	3.0 (0.6-5.4)			The buffalo samples were collected at three abattoirs in Ho Chi Minh City 2–5-years-old, originating from different parts of the country

IV.3. Seroprevalence of *T. gondii* in humans

The disparity of toxoplasmosis seroprevalence in animals was also found in humans among different countries in Southeast Asia. This may due to the different serological techniques used, population study groups, study areas and risk factors. As an example of the limitation of seroprevalence rates due to the use of different serological techniques, a study in Thailand showed a large disparity of seroprevalence using 3 different serological techniques on the same population, Sabin Feldman Dye Test, ELISA kit 1 and ELISA kit 2: 13.0%, 34.7% and 36.3% respectively (Chemoh et al., 2013). Overall, the seroprevalence rate ranged from 3.1% to 70.0%. High seroprevalence was found mainly in 3 countries, Indonesia, Malaysia and Philippines, whereas Singapore, Thailand and Vietnam exhibited low seroprevalence (Table 5).

Seroprevalence studies were classified in different population groups for better understanding.

VI.3.1. Pregnant women

The seroprevalence rate ranged from 5.7% in Thailand (Wanachiwanawin et al., 2001) to 49.0% in Malaysia (Nissapatorn et al., 2003c). High seroprevalence in Malaysia was found associated with potential risk factors such as presence of owned or stray cats at home, consumption of undercooked meat, drinking unpasteurized milk, drinking untreated water and contact with soil (Nissapatorn et al., 2003c). The lack of awareness of toxoplasmosis was one of the factors contributing to high seroprevalence in pregnant women. A study in Thailand showed a high seroprevalence (53.7%) in pregnant women HIV infected (Wanachiwanawin et al., 2001). The majority of subjects were poor, low educated employees. The low socio-economic level, usually associated with poor hygienic conditions, represents a higher risk of contamination through environment. It may explain the higher prevalence compared to other studies in pregnant women in this country.

IV.3.2. HIV infected and AIDS patients

High seroprevalence was found in Indonesia and Malaysia, 41.6% (Sari et al., 2015) and 51.2% (Nissapatorn et al., 2003b) respectively. *Toxoplasma*-infected AIDS patients are at risk of *T. gondii* reactivation. The high seroprevalence was suggested to be due to socioeconomic status and risk behaviors of the patients (Nissapatorn et al., 2003b).

IV.3.3. Inhabitants (general population)

A high seroprevalence was observed in Malaysian and Indonesian people, 59.7% (Ahmad et al., 2014) and 70.0% (Terazawa et al., 2003) respectively but the seroprevalence found in Vietnam was quite low with 4.2% (Udonsom et al., 2008). The low seroprevalence could due to the hygiene conditions that have been improved in the country and probably related to the culinary habits of Vietnamese population who eat meat generally well cooked (Udonsom et al., 2008; Buchy et al., 2003). The culinary habit of Indonesian population may cause a potential risk to *T. gondii* infection (Matsuo and Husin, 1996). Meat was generally not adequately cooked. Another risk factor may influence in this high seroprevalence such as contact with soil (Konishi et al., 2000). The majority of Malay work as fishermen and employees, their houses are poorly constructed, the kitchens and bathrooms of some houses were observed to be in poor condition (Ahmad et al., 2014). Once again, lack of hygiene and cleanliness could be an important source of infection. In Philippines, the disparity of seroprevalence was found between urban and rural areas, 11.0% and 61.2% respectively (Kawashima et al., 2000). The lifestyle of people who live in urban areas is different from people who live in rural areas. Regarding people living in rural areas, the main occupation is agriculture. They are always in contact with soil and animals. The sanitation system is not adequately developed and sometimes people have to drink the water directly from the river. People could be contaminated from *T. gondii* oocysts containing in the water. Concerning people who live in urban areas, the sanitation system is better developed than rural areas explaining lower *T. gondii* seroprevalence. Low seroprevalence of toxoplasma infection was found in Thai population. Thailand is moving from agriculture base to industrial base. The socio-economic level is more developed than in the last 3 decades. The sanitary system is well organized even in remote areas. Risk factors such as hygiene problem and the poverty leading to the contamination by several parasites decreased according to better lifestyle conditions.

IV.3.4. Blood donors

In Thailand, a low seroprevalence was observed in blood donors: 3.1% in Bangkok (Maruyama et al., 2000) and 9.6% in Loei (Pinlaor et al., 2000). Loei is situated in Northeastern region of Thailand. Although people living in this region are reputed for consuming raw meat and fish, the seroprevalence remained quite low. This might due to the low seroprevalence found in the common consumption animals.

IV.3.5. Other population groups

1. Orang Asli Arborigine (ethnic group in Malaysia)

The seroprevalence varied from 10.6% (Hakim et al., 1994) to 37% (Ngui et al., 2011). They live in the closest possible association with tropical forest. Seroprevalence of *T. gondii* was in relation to several risk factors such as lack of education, use of untreated river and mountain water supplies and close contact with pets (Ngui et al., 2011).

2. People having close contact with animals

The seroprevalence rate ranged from 6.4% in Thailand (Sukthana et al., 2003) to 19.9% in Malaysia (Brandong-Mong et al., 2015). A higher seroprevalence was found in veterinary technicians compared to other population groups, this could due to the exposure with animals, especially cats (Brandong-Mong et al., 2015).

3. Farmers

The seroprevalence found was 27.8% in Malaysian farmers (Normaznah et al., 2004). It was suggested that the farmers probably acquired the infection from the cats that may frequent their premises and from contact with soil.

4. Various groups of patients

In Malaysia, 56.7% of seroprevalence was found in patients with kidney failure, 21 to 89 years old (Nissapatorn et al., 2011). It seems quite high as in the general population of Malaysia. *Toxoplasma* seroprevalence was significantly associated with several factors such as age group, race, marital status, level of education and type of renal diseases.

As *Toxoplasma* infection was described in many studies in the world as associated to an increased risk of schizophrenia, two studies, both in Malaysia tried to find this link in Southeast Asian populations. The seroprevalence ranged from 37.5% (Emelia et al., 2012) to 51.5% (Omar et al., 2015) in Malaysia. In this last study, it was higher than in a control population study suggesting a possible association with this neurological condition.

Conclusion: Although seroprevalence studies are still limited in Southeast Asian countries, it seems that the overall seroprevalence in humans was quite low in Southeast Asian countries except for Indonesia and Malaysia, which are situated in equatorial zone. The equatorial climate may increase the *T. gondii* infection rate as high seroprevalence observed in South America and Africa (see figure 14). This low seroprevalence have been suggested to be due to tropical climate. However, this hypothesis is contradicted by the high seroprevalence found in Africa, up to 75.2% in Sao Tome, and South America up to 77.5% in Brazil (Pappas et al., 2009). Generally, oocysts of *T. gondii* are very resistant to hard conditions as the dryness and the heat but for example, in Thailand, in the hottest season, the temperature may exceed 40°C. What could explain the different rates of prevalence between these groups of countries in the similar climatic environment? There is still no clear answer. As shown in our review, seroprevalence in animals used for consumption did not show fundamental differences between countries. The culinary habit of Southeast Asian populations may be questioned for analyzing this seroprevalence of *T. gondii*. Socioeconomic condition might be involved in the parasite transmission as lack of hygiene and low education. Occupation may have an influence on the risk of infection. Even if the industry begins to spread in the Southeast Asian countries, a large part of the population are living and working in rural areas where contact with soil and animals represents a risk factor. Domestic animals become very common in almost families especially cats, dogs and some pet birds. Stray cats and dogs were found in a lot of monasteries and also in the streets.

The studies concerning toxoplasma seroprevalence are limited in Southeast Asian countries. There are no study in Brunei and East Timor and there were only one or 2 studies in some countries. More studies are needed in Southeast Asian countries in order to better understanding the circulation of *T. gondii* in the populations of these countries.

Toxoplasmosis seroprevalence in equatorial region (Malaysia and Indonesia: dark blue areas) is higher than in tropical monsoon region (Thailand, Vietnam, Laos and Cambodia: light blue areas) (Figure 14).

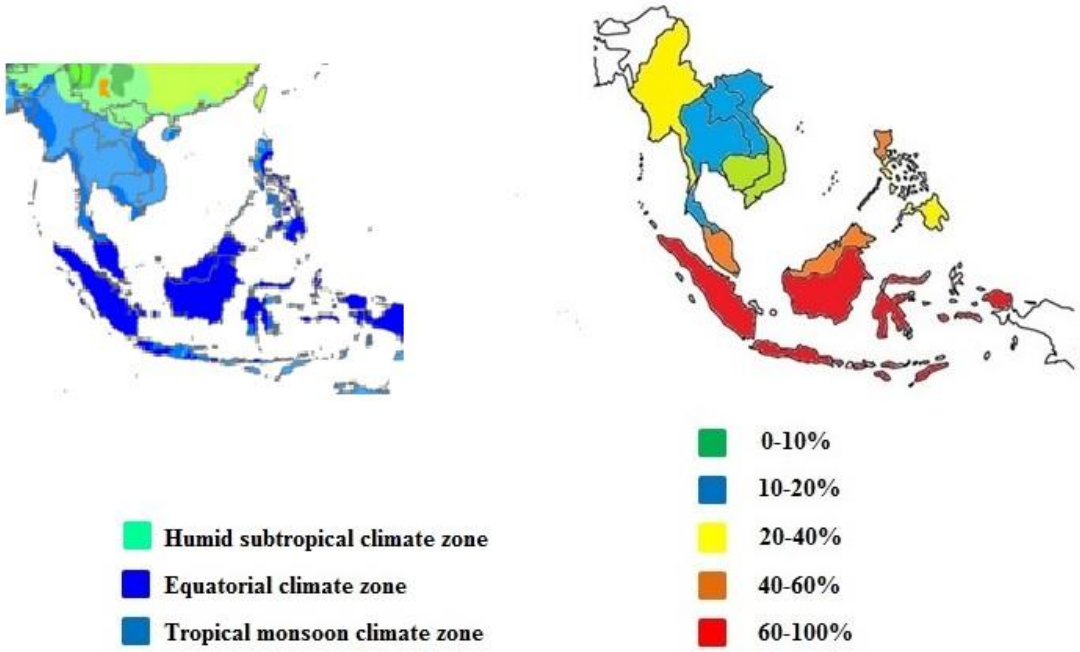


Figure 14: Toxoplasmosis seroprevalence in general population in Southeast Asian countries compared to different climatic conditions.

Table 5: *Toxoplasma gondii* seroprevalence in humans in Southeast Asian countries.

Countries	Population	Sample size	Age (years)	Prevalence (% and 95% confidence interval	Technique	References	Comments
Cambodia							
Whole country	Women child bearing age	2150	15-39	5.8 (4.7-7.0)	In house ELISA Recombinant antigen SAG2A	Priest et al., 2016	
Indonesia							
Surabaya, urban areas	General patients at the Emergency unit	1761	0-100	58.0 (55.7-60.3)	In house ELISA	Konishi et al., 2000	Higher seroprevalence in males than in females
East Java	Inhabitants who visited Sidoarjo city hospital	244	1-80	64.0 (58.0-70.0)	LAT (Toxo-test Eiken kit)	Uga et al., 1996	97 patients complained of diarrhea and 147 persons visited the hospital for routine medical
Jakarta	Inhabitants	1693	20-85	70.0 (67.8-72.2)	In house ELISA	Terazawa et al., 2003	Seroprevalence was almost the same in females and in males
Java	HIV patients	122	-	41.6 (37.3-45.9)	ELISA kit DRG international	Sari et al., 2015	
	Inmates from 4 prisons	375					For participants in the 4 prisons, the injection use was associated with *T. gondii* antibodies
Laos							
Keoudom, Vientiane, rural areas	Workers of hydroelectric station and their family Inhabitants of neighboring villages Patients of the Institute of Malaria and Parasitic diseases in Vientiane	640	3-70	15.3 (12.5-18.1)	CFT	Catar et al., 1992	Seroprevalence increase with an increasing age
Malaysia							
Klang Valley	People having close contact with animals; veterinarian, technician, student and pet owner	312	17-64	19.9 (15.5-24.3)	ELISA kit (IgG-NovaLisa)	Brandong-Mong et al., 2015	High seroprevalence in veterinary technicians Gardening was a risk factor for *T. gondii* infection

Pangkor Island, Peninsular	Inhabitants also in ethnic groups, Malay, Chinese and Indian	298	1-80	59.7 (54.1-65.3)	ELISA kit (Trinity Biotech)	Ahmad et al., 2014	Higher seroprevalence in females than in males High seroprevalence among the Malays followed by Indians and Chinese
Attending Sungai Buloh Hospital, Selangor	Patients with schizophrenia	101	18-65	51.5 (41.8-61.2)	ELISA kit (IBL Company)	Omar et al., 2015	Higher seroprevalence in schizophrenia than in controls
	Healthy individuals as controls	55	21-63	18.2 (8.0-28.4)			
Malaya hospital	Pregnant women visiting the antenatal clinic	219	20-41	42.47 (36.11-49.09)	ELISA kit (IgG-NovaLisa	Andiappan et al., 2014b	High seroprevalence in pregnant women in the age group of 30 years and above
Kuala Lumpur	Patients with schizophrenia	144	21-40 and 40 >	37.5 (29.6-45.4)	ELISA kit (Platelia Toxo IgG ELISA BioRad)	Emelia et al., 2012	No statistical difference between seroprevalence rate in schizophrenia patients and psychiatrically healthy
	Healthy volunteers	144	21-40 and 40 >	34.0 (26.3-41.7)			volunteers
Peninsular	Orang Asli Indigenous	495	1-82	37.0 (32.7-41.3)	ELISA kit (IgG and IgM; Trinity Biotech)	Ngui et al., 2011	Participants having close contact with pets were 2.7 times more likely to acquire toxoplasmosis
Peninsular, Gombak hospital	Orang Asli (arborigine)	415	4-50 and 50 >	10.6 (7.6-13.6)	IFAT	Hakim et al., 1994	Slightly higher seroprevalence in females than in males
Kuala Lumpur, Malaya medical centre	Renal patients	247	21-89	56.7 (50.5-62.9)	ELISA kit (IgG and IgM; Trinity Biotech)	Nissapatorn et al., 2011	Age group, race, marital status, level of education, and type of renal diseases were significantly associated with Toxoplasma
Gombak District, Selangor	Farmers	79	-	27.8 (17.9-37.7)	IFAT	Normaznah et al. , 2004	Seroprevalence rate may due to the degree of exposure to the source of infection
Malaya medical centre	Pregnant women gestation periods ranging from 7 to 39 weeks	200	18-43	49.0 (42.1-55.9)	ELISA kit (Trinity Biotech, Bray)	Nissapatorn et al., 2003c	No significant association with risk factors
Kuala Lumpur, hospital	AIDS patients	406	17-74	51.2 (46.3-56.1)	ELISA kit (AxSYM, USA)	Nissapatorn et al., 2003b	High seroprevalence was found Toxoplasmosis is still a major

							problem worldwide particularly in HIV/AIDS patients
Kuala Lumpur, hospital	AIDS patients	301	18-78	41.2 (35.6-46.8)	ELISA kit (Trinity Biotech)	Nissapatorn et al., 2003a	Higher seroprevalence in the Malays than in other ethnic groups
Oil palm plantation	Foreigner migrant workers	501	-	34.1 (29.9-38.3)	IFAT	Chan et al., 2008	Seroprevalence variation due to differences in dietary habits behavioral risks, environmental conditions and hygiene
	Local Malaysian workers	198	-	44.9 (38.0-51.8)			
Myanmar							
Yangon Central Women Hospital	Pregnant women	215	18-45	30.7 (27.92-37.16)	ELISA kit (IgG-NovaLisa)	Andiappan et al., 2014b	Lack of awareness of toxoplasmosis was one of the factors contributing to high prevalence in these pregnant women
Philippines							
Manila	Inhabitants urban areas	908	1-50 and 50 >	11.0 (9.0-13.0)	ELISA (in house)	Kawashima et al., 2000	In Manila, prevalence of Toxoplasma antibody increased with age
Mindoro	Inhabitants rural areas	152	1-50 and 50 >	61.2 (53.5-68.9)			
Leyte	Inhabitants rural areas	113	1-50 and 50 >	30.1 (21.6-38.6)			
Manila	Inhabitants urban areas	68	16-56	23.5 (13.4-33.6)	LAT (TOXOCELL AD Direct Agglutination Test Kit)	Salibay et al., 2008	Slightly higher seroprevalence in suburban area than in urban area
Dasmariñas,Cavite	Inhabitants suburban areas	72	16-56	30.6 (20.0-41.2)			
Singapore							
Clinic in KK women's and children's hospital	Pregnant women	120	≤ 30 ≥	17.2 (10.4-24.0)	Toxoplasma Ab IgG immunofluorescent test	Wong et al., 2000	The racial distribution of populations were Chinese (81.7%), Indians (1.7%), Malay (13.3%) and other races (3.3%)

Thailand

Antenatal clinic (ANC), Songklanagarind Hospital, Hat Yai	Pregnant women	640	15-45	28.3 (24.8-31.8)	Elisa kit (IgG-Trinity Biotech and IgM-Trinity Biotech	Nissapatorn et al., 2011	Risk factors to Toxoplasma acquisition is careless when preparing food that can lead to contamination
Bangkok	Cat owners	327	20-59	6.4 (3.7-9.1)	Sabin-Feldman Dye Test	Sukthana et al., 2003	Household members,monks, novices and nuns were enrolled
Bangkok, Siriraj Hospital	Pregnant women HIV infected	838	-	53.7 (50.3-57.1)	ELISA kit (Platelia® Toxo IgG, Sanofi Diagnostics Pasteur)	Wanachiwanawin et al., 2001	Significantly higher seroprevalence in pregnant women HIV infected than in pregnant women non HIV infected
	Pregnant women non HIV infected	831	-	5.7 (4.1-7.3)			
Bangkok	Blood donors Pregnant women Kidney recipients HIV infected persons	200 100 100 100	16-67	16.0 (12.8-19.2) 11.4 (8.6-14.2)	Direct agglutination kit (Toxo-Screen DA, bioMerieux), Sabin Feldman Dye Test	Sukthana et al., 2001	ToxoScreen was more sensitive than Sabin Feldman Dye Test
Loei province, northeast	Blood donors	345	17-56	9.6 (6.5-12.7)	ELISA (in house)	Pinlaor et al., 2000	Significantly higher seroprevalence in males than in females
Bangkok	Blood donors	163	2-89	3.1 (0.4-5.8)	LAT kit (Toxocheck, Eiken)	Maruyama et al., 2000	No statistical difference was observed between males and females
Samut Sakhon, ANC clinic of Samut Sakhon general hospital	Pregnant women	300	14-41	21.7 (17.0-26.4)	Sabin-Feldman Dye Test	Sukthana, 1999	Low seroprevalence was found
Samut Sakhon	Pregnant women HIV infected Pregnant women non HIV infected	1200	< 20-40 >	21.1 (18.8-23.4) 13.1 (11.2-15.0)	Sabin-Feldman Dye Test	Chintana et al., 1998	Significantly different between properly and undercooked meat consumers. Significant difference between pregnant women who had cat and no cat in their houses

Songkhla, Songkhlanagarind Hospital, Hat Yai	Pregnant women	450	-	14.7 (11.4-18.0) 29.6 (25.4-33.8) 38.7 (34.2-43.2)	Sabin-Feldman Dye Test ELISA kit 1 ELISA kit 2	Chemoh et al., 2013	No significant difference of seroprevalence between pregnant women and HIV subjects using three different serological diagnostic tests
	HIV infected patients	300	-	13.0 (9.2-16.8) 34.7 (29.3-40.1) 36.3 (30.9-41.7)			
Songkhla, Songkhlanagarind Hospital, Hat Yai	Pregnant women	760	14-47	25.0 (21.9-28.1)	ELISA kit (gG-T and IgM-Trinity Biotech)	Andiappan et al., 2014a	Low-socioeconomicand poor hygiene practice can play as an important role in the transmission of the parasites
Vietnam							
3 provinces Nghe An, Lao Cai and Tien Giang	Vietnamese villagers	650	1-61 >	4.2 (2.7-5.7)	Sabin Feldman Dye Test	Udonsom et al., 2008	Consumption of undercooked or raw meat was rarely found in this group
Ho Chi Minh Nha Trang	Drug user HIV infected Pregnant women	300 300	18-53 18-43	7.7 (4.7-10.7) 11.2 (7.6-14.8)	Platelia Toxo IgG and IgM, Bio-Rad	Buchy et al., 2003	Low seroprevalence in drug users and in pregnant women probably related to the culinary habits Vietnamese people eat meat generally well cooked.

V. Clinical aspects of human toxoplasmosis in Southeast Asia

In Southeast Asian countries, there are few studies on clinical forms of toxoplasmosis. We found no studies concerning clinical forms of toxoplasmosis in Brunei, Cambodia, East Timor, Laos, Myanmar, Philippines and Vietnam. Some studies conducted in Indonesia, Malaysia, Singapore and Thailand give some insight on the consequences of *T. gondii* infection in Southeast Asian countries. Overall, 21articles were retained for this review (Table 6).

V.1. Acquired toxoplasmosis

We found only one study reporting that 11% of lymphadenopathies cases were due to toxoplasmosis in Malaysia (Tan et al., 1978, cited in Tan and Mak, 1985). Associated symptoms were, as classically reported, sore throat, fever, and mononucleosis. In Malaysia, Suhardjo et al., (2003), in their cohort of 173 patients with retinochoroiditis, reported 3 cases of acquired toxoplasmosis with neuroretinitis, lymphadenopathies and fever. Other cases of ocular toxoplasmosis in the context of acquired toxoplasmosis will be described in a separate paragraph.

No report of disseminated forms with pulmonary involvement or other organ failure as described in South America was found.

V.2. Congenital toxoplasmosis

In the absence of systematic screening of congenital toxoplasmosis in Southeast Asian countries, it is difficult to have reliable data on incidence and prevalence rate. However, the estimation of congenital toxoplasmosis incidence was estimated at 1.3 per 1,000 live births in Southeast Asian Region as defined by World Health Organization (Torgerson and Mastroiacovo, 2013). In a pilot screening study to detect congenital infection, Sukthana et al., 2005 (cited by Pengsaa and Hattasingh, 2015) analyzed sera for IgM antibodies using a commercially immunocapture assay (ELISA) kit in 8064 neonates delivered at Rajvithi Hospital, Bangkok, between February 2001 and March 2002. No IgM antibody was found in the neonates during the study period, and the seroconversion rate was 2/1000 pregnancies, suggesting that *Toxoplasma* congenital infection is a rare occurrence in this country. In Malaysia, using a less sensitive assay (IFAT), IgM were detected in 2% of 405 apparently healthy children aged 0-4 month old (8/405), and in only 0.3% of 1060 defective children (Tan and Mak, 1985). These defective children were defined as having neonatal jaundice, hepatomegaly, hydrocephaly, microcephaly and ocular lesions for children aged 0-4 month-

old, and mental retardation for children (1-4 years old). IgM antibodies indicating a possible congenital infection were detected in 1 out of 312 (0.3%) of neonatal jaundice cases, 3/627 (0.5%) of hepatomegaly cases, 1/73 (1.4%) of microcephaly cases. No IgM positive cases were found in children with hydrocephaly (0/34), ocular lesions (0/14), and mental retardation (0/79). The authors concluded that *T. gondii* is not an important parasite that causes congenital disease in Malaysia (Tan and Mak, 1985). This conclusion may be nuanced, as IFAT IgM is known to be positive at birth in only 25% of cases of congenital toxoplasmosis and as the disappearance of IgM in a few months after birth (Robert-Gangneux and Dardé, 2012) may explain their absence in 1-4 years-old mentally retarded children.

In the same Malaysian study of Tan and Mak (1985), it was noted that one third of detected cases of congenital toxoplasmosis were asymptomatic. Clinical features of congenital toxoplasmosis in these countries are mainly known through clinical cases reports that usually corresponded to the most severe symptomatic forms. The classical neuro-ocular forms are the most frequently reported, although disseminated forms were also detected.

In Indonesia, an infant 15 day old with a high *T. gondii* antibody with ELISA test (IgG positive but IgM negative) presented with symptoms suspected to be congenital toxoplasmosis: hydrocephalus, cerebral calcification, and chorioretinitis (Harun et al., 1989). Another case in Indonesia, an 18 month old child with blindness, microphtalmia and bilateral cataract symptoms was identified. The serological results of this child and his mother were positive for the 3 serological tests performed, IHAT, IFAT and Sabin Feldman Dye Test (Partono and Cross, 1976). Although in these 2 cases, serological tests were insufficient to ascertain the diagnosis, congenital toxoplasmosis is highly probable.

In Singapore, 3 cases of congenital toxoplasmosis were detected among 1848 mentally-retarded children. One of them presented a particularly severe form with a severe mental retardation, microcephaly and blindness (Paul, 1982).

In Thailand, in a retrospective study using questionnaires and literature search between 1995-2013, congenital toxoplasmosis was diagnosed in thirteen patients and highly suspected in 7 patients (Pengsaa and Hattasingh, 2015). Only 5/20 children presented the classical classic triad of hydrocephalus, cerebral calcification and chorioretinitis. But, even if the triad was not complete, these neuro-ocular signs were present in the majority of children: 14/20 retinochoriditis, 12/20 intracranial calcifications, 10/20 hydrocephalus. But what is remarkable is that nearly all of them (19/20) had manifestations of systemic infection (hepatosplenomegaly, thrombocytopenia). This high rate of disseminated infection may reflect a selection bias due to recruitment through questionnaires. Fifteen patients survived

and 5 died. Deafness, visual impairment and delayed development were neurological sequelae in the 15 surviving patients (Pengsaa and Hattasingh, 2015).

Finally, three studies performed in referral eye clinics, one in Malaysia (Lim and Tan, 1983), and two in Indonesia (Suhardjo et al., 2003) described clinical aspects of ocular lesions in suspected congenital cases. In the cohort of 170 cases in Indonesia, patients presented with chorioretinitis (71.2%), macular scars (22.4%), squint (6.4%), congenital cataract (2.8%), nystagmus (6.4%) and atrophic optic papilla (2.8%). Lesions were bilateral in 34% of cases. Neurological impairment was associated in 4 cases: intracerebral calcifications (2 cases), hydrocephalus (1 case), and encephalitis (1 case in 23-year-old woman who died). The other Indonesian study was carried out in 41 children with ocular lesions under 12 years of age. Clinical symptoms and positive serological tests (IFAT and ELISA) were used to diagnose the ocular toxoplasmosis. From 41 children, 28 out of them were screened positive to *T. gondii*. Among 28 *T. gondii* positive children, 14/28 (50.0%) had bilateral involvement, 9/28 (32.0%) showed unilateral involvement for the right eye and 5/28 (18.0%) had unilateral involvement in the left eye (Kadarisman et al., 1991).

V.3. Toxoplasmosis in immunocompromised patients

Symptoms of cerebral toxoplasmosis reported in immunosuppressed patients (mainly AIDS patients), in Southeast Asia do not present any peculiarity. In the different published studies, diagnosis is made through neuroimaging and response to sulfonamides-pyrimethamine treatment.

Three studies were case reports from Malaysia:

- In a 31 year old HIV-positive man, cerebral toxoplasmosis was confirmed by MRI scan which showed the multiple cerebral lesions and by a serological test (ELISA) with a high positive titer 300 IU/ml (Nimir et al., 2013).
- In three cases of HIV infected persons, neuroimaging demonstrated obstructive communicating hydrocephalus and serological tests were positive for *T. gondii* (Basavaprabhu et al., 2012).
- An 18 year old male Malaysian student have Systemic Lupus Erythematosus (SLE) and 4 days later, the result of CT scan on his brain showed the multiple lesions suggesting a cerebral toxoplasmosis (Pagalavan and Kan, 2011).

Retrospective or cross-sectional studies in HIV infected patients were reported in Thailand and Indonesia.

- In Indonesia, patients with meningismus, headache, lowered consciousness, fever, hemi or tetraparesis, cranial nerve palsies and seizures were included in this study, most of them are HIV infected. *Toxoplasma* IgG was found in 78% of patients. *T. gondii* PCR was used for diagnosis of cerebral toxoplasmosis and 32.8% of patients were screened positive. Mortality rate was 2.16 fold higher in patients positive CSF *T. gondii* PCR than in patients negative CSF *T. gondii* PCR (Ganiem et al., 2013).
- One hundred and fifty-five HIV patients were enrolled in the study in Chiang Mai university, Thailand. Neurological implication was 50.3 per 100 person-years. The incidence of cerebral toxoplasmosis was 14.8 per 100 persons-years. Cerebral toxoplasmosis was a common opportunistic infection in AIDS patients. The incidence of cerebral toxoplasmosis appeared to be increasing although after the HAART treatment (Subsai et al., 2004).
- At Manorom Christian Hospital Infectious Disease Clinic, Chainat, Thailand, among 207 HIV patients with available data, 8/207 (4.0%) had clinical features of cerebral toxoplasmosis (Inverarity et al., 2002).
- In HIV patients from Ramathibodi Hospital, Bangkok, Thailand, CT scan technique was used to diagnose the neurological involvement. All patients presented abnormal CT results and cerebral toxoplasmosis was confirmed in 42 out of 195 patients (22.0%) with the presence of nodular and multiple brain lesions (Hongsakul and Laothamatas, 2008).

V.4. Ocular toxoplasmosis

Diagnosis of ocular toxoplasmosis is commonly based on the characteristic clinical findings, associated to a positive *Toxoplasma* serology. The additional examinations (polymerase chain reaction and/or Goldmann–Witmer coefficient) that would ascertain the diagnosis necessitate sampling of ocular fluid, and are rarely available in publications from these countries (Kongyai et al., 2012). Similarly, distinction between congenital and acquired cases of ocular toxoplasmosis is not easily performed.

The majority of studies were prospective or retrospective studies of cases of uveitis addressed to referral eye centers. This does not allow having an idea of the incidence of ocular involvement during acquired toxoplasmosis. But, all these studies confirm that ocular toxoplasmosis is the most common infectious cause of uveitis in Northern Thailand (Pathanapitoon et al., 2008; Sirirungsi et al., 2009), in Southern Thailand (Sittivarakul et al., 2013), in Singapore (Phaik et al., 1991) or Indonesia (Kadarisman et al., 1991; Suhardjo et al.,

2003). The most frequent anatomical lesions are panuveitis or posterior uveitis with focal retinochoroiditis. Retinal scars may be associated mainly in congenital cases.
Apart the clinical signs, the possible *Toxoplasma* origin of the ocular lesion is suggested by the observation of a higher rate of *Toxoplasma* prevalence in patients with uveitis.

One hundred and thirty patients with ocular inflammation were enrolled in the study in Malaysia. ELISA serological test was used to detect *T. gondii* IgG and IgM antibodies, 71 patients out of 130 were positive retinal scars were more frequent in patients with a positive *Toxoplasma* serology, and 18 were diagnosed as having an active ocular toxoplasmosis according to the clinical features (Suresh et al., 2012). At the ophthalmology clinic of University Kebangsaan, Kuala Lumpur in Malaysia, 31 cases of ocular toxoplasmosis were observed. Typical clinical findings, positive serological titer of 1: 64 or more and exclusion of other common causes of retinochoroiditis were used to diagnose ocular toxoplasmosis. The most common symptoms found in these 31 ocular toxoplasmosis was blurred vision (Lim and Tan, 1983).

The IFAT to *T. gondii* antibody was performed to diagnose 122 uveitis patient serums in Singapore. The result of IFAT showed 28.7% of *T. gondii* seropositivity and 35 eyes from 29 patients were confirmed to be ocular toxoplasmosis (Phaik et al., 1991).

In Chiang Mai university hospital in Thailand, 25 focal chorioretinitis patients were included in the study to identify the *T. gondii* antibodies. Elisa test was used and the result showed 21/25 (84%) of FCR patients had *T. gondii* antibodies. *T. gondii* could be the parasite, which cause ocular toxoplasmosis in Thailand (Pathanapitoon et al., 2013). The study was carried out at the Uveitis and Ocular Inflammatory Disease Clinic, Department of Ophthamology, Songklanagarind Hospital, Songkla, Thailand. In total, 254 patients were enrolled. Ocular toxoplasmosis was diagnosed in 18/254 (7.1%) cases (Sittivarakul et al., 2013). At the Department of Ophthalmology, Chiang Mai university, Thailand, sixty-one consecutive posterior uveitis patients were included to observe the presence of *T. gondii*. Specific antibodies to *T. gondii* was detected by Goldmann–Witmer coefficient (GWC) analysis and only 1/61 (1.6%) of patients was found positive (Kongyai et al., 2012). A study on 101 new consecutive non HIV infected with uveitis patients, 100 HIV infected with retinitis patients and 100 non uveitis as control group were enrolled and the study was carried out at the ophthalmology department of Chiang Mai University Hospital, Thailand. The seroprevalence found in these 3 groups were 31/101 (31.0%), 19/100 (19.0%) and 17/100 (17.0%). A higher seroprevalence to *T. gondii* was found in non HIV infected with uveitis patients that may suggest the important role of *T. gondii* in ocular toxoplasmosis (Sirirungsi et

al., 2009). The study was also conducted at the Department of Ophthalmology, Chiang Mai university, Thailand. Two hundred consecutive uveitis subjects were included, 138 out of them were non HIV infected and 62 subjects were HIV infected. One of the most common infections was ocular toxoplasmosis with 12/138 (8.7%) in non-HIV infected uveitis subjects (Pathanapitoon et al., 2008).

Table 6: Clinical aspects of Human toxoplasmosis in Southeast Asia.

Country	Population	Study design	Clinical form	Clinical signs	Diagnostic criteria	References
Indonesia	41 children < 12 years old	Retrospective	Ocular	Visual impairment, blindness (19.0%), strabismus or nystagmus, bilateral lesions (50.0%)	clinical and serological criteria (toxo +)	Kadarisman et al., 1991
Indonesia	3 cases of acquired toxoplasmosis (neuroretinitis, lymphadenopathies, fever) - 19-23 years old	Retrospective cohort	Ocular/congenital	- Chorioretinitis (71.2%), macular scars (22.4%), squint (6.4%), congenital cataract (2.8%), nystagmus (6.4%) and atrophic optic papilla (2.8%). - Intracerebral calcification (2 cases), hydrocephalus (1 case), encephalitis (1 case in 23-year-old woman who died)	Clinical and serological criteria (toxo +) (IgM for cases of congenital toxoplasmosis)	Suhardjo et al., 2003
Indonesia	HIV 64 HIV-infected patients (median CD4 22)	Retrospective	Cerebral	Sub acute meningitis	Neuroradiology not available	Ganiem et al., 2013
Malaysia	Systemic lupus erythematosus treated with methylprednisolone	Case report	Cerebral	Fever, seizures, multiple cerebral abcesses	Neuroimaging, response to treatment (clindamycin plus pyrimethamine)	Pegalavan and Kan, 2011
Malaysia	AIDS patient	Case report	Cerebral	Seizures, multiple cerebral abcesses	Neuroimaging, response to treatment (sulfonamides plus pyrimethamine)	Nimir et al., 2013
Malaysia	AIDS patients	Case report (3 cases)	Cerebral	Obstructive hydrocephalus, brain abscess	Neuroimaging, response to treatment (sulfonamides plus pyrimethamine)	Basavaprabhu et al., 2012
Malaysia	161 patients with lymphadenopathies and mononucleosis	Retrospective cohort	Acquired	Lymphadenopathies, fever, sorethroat, and mononucleosis		Tan et al., 1978
Malaysia	130 patients with ocular inflammation [38.41 (range 6–83) years]	Retrospective review	Ocular	- Chorioretinal scar more frequent ($p<0.05$) in patients toxo positive - 18 patients with active ocular toxoplasmosis (13.8%)	Clinical and serological criteria (Platelia ELISA, toxo + , IgG IgM, avidity)	Suresh et al., 2012
Malaysia	31 cases of ocular toxoplasmosis, 14 to 68 years old	All consecutive cases of ocular toxoplasmosis	Ocular/congenital	- Visual impairment bilateral in 50% of cases - Pigmented macula scar indicative of a chronic infection - 12 with acute recurrences	Clinical and serological criteria (toxo + IFAT) (IgM for cases of congenital toxoplasmosis)	Lim and Tan, 1983
Malaysia	Children 0-4 months old	Cohort of 1465 children (405 apparently healthy and 1060 defective children	Congenital	- 1/3 asymptomatic cases - Microcephaly (1), hepatomegaly (3), jaundice (1) - No case of hydrocephalus or ocular lesions	Toxo IgM pos (IFAT)	Tan and Mak, 1985

Singapore	Children	Retrospective review of mental retardation (1848 cases) in a pediatric center	Congenital	3 cases (microcephaly, spasticity)		Paul, 1982
Singapore	122 adult patients with uveitis	Retrospective	Ocular	- Whitish yellow, and fuzzy outlined lesion of the retina and choroid, adjacent to a previous chorioretinal scar, associated with vitritis and vasculitis - Bilateral uveitis in 6 cases	Clinical and serological criteria (Toxo + IgG)	Phaik et al., 1991
Thailand	HIV/AIDS all adults HIV-infected newly diagnosed (207) in a rural healthcare facility in Central Thailand	Cross-sectional	Cerebral	Recent onset of focal neurological abnormality or reduced level of consciousness	Neuroimaging, response to treatment (sulfonamides plus pyrimethamine) Serology not available	Inverarity et al., 2002
Thailand	HIV/AIDS	Retrospective study of the incidence of AIDS-defining diseases in the patients admitted to Chiang Mai (155 cases)	Cerebral		Neuroimaging and high antibody titers	Subsai et al., 2004
Thailand	HIV/AIDS adult HIV patients referred for CT scan at Ramathibodi Hospital (Bangkok) (195 cases from 2001 to 2005)	Retrospective	Cerebral		CSF analysis, pathological report, and response to treatment	Hongsakul and Laothamatas, 2008
Thailand (North)	200 consecutive new patients with uveitis in an ophtalmologic clinic	Prospective case series	Ocular	- 10 focal retinitis - 2 non-HIV patients with focal active chorioretinitis located adjacent to a chorioretinal scar	Full ophthalmic examination, serology response to treatmennt in case of active lesion	Pathanapitoon et al., 2008
Thailand (North)	101 consecutive patients with uveitis, 100 HIV-infected retinitis patients, and 100 nonuveitis controls	Prospective case	Ocular	Posterior uveitis had the highest prevalence of positive *T. gondii* serology results compared to other anatomical types of uveitis	Serology	Sirirungsi et al., 2009
Thailand (North)	80 consecutive patients with posterior uveitis (n = 38) and panuveitis (n = 42) of	Prospective case	Ocular	- 3 focal retinitis - 1 with retinal vasculitis without any focal retinal lesions	PCR in ocular fluid Goldmann–Witmer coefficient (GWC)	Kongyai et al., 2012

	unknown origin				analysis for *T. gondii.*	
Thailand (North)	25 consecutive patients with focal retinitis 127 patients with uveitis without focal retinitis (FCR)	Prospective case	Ocular	- Active single chorioretinal lesion with diameter of >4 disk diameters, suggesting a primary acquired infection - Absence of old scars and retinal arteritis, distinctive feature from typical OT lesions in the European and the U.S. series	Serology level of antibody titers	Pathanapitoon et al., 2013
Thailand (South)	254 consecutive cases of uveitis refered to an ophtalmologic center	Prospective case	Ocular	Posterior uveitis, panuveitis	Serology, investigations of oher causes	Sittivarakul et al., 2013
Thailand	Children	Retrospective study: questionnaire during 1995–2013 (paediatricians in referral and university hospitals) and literature review (20 cases)	Congenital	- 19/20: systemic manifestations (hepatosplenomegaly,thrombocytopenia) - 5/20: classic triad of hydrocephalus, cerebral calcification and chorioretinitis. - 14/20: chorioretinitis - 12/20: intracranial calcifications - 10/20: hydrocephalus - 5 deaths: 1 at 36 h post natal with respiratory distress, hepatic involvement, 1 at 13 years old with a pulmonary infection.	IgM antibodies and/or clinical signs	Pengsaa and Hattasingh, 2015

Conclusion: In Southeast Asian countries, as well as in many other tropical areas, human toxoplasmosis and its clinical aspects has long been a neglected subject. The low prevalence observed in many of these countries, notably in Thailand, may also explain the relative lack of clinical research in this field. From our literature review, there is a lack of reports in many countries of this area (Vietnam, Cambodia, Laos, Myanmar, Brunei, East Timor, Myanmar and Singapore). Even if we have some data on toxoplasmosis in Thailand, Indonesia and Malaysia, data are still lacking especially in remote areas of these countries.

The AIDS epidemic has focused attention of clinicians on cerebral toxoplasmosis cases associated to reactivation. Due to the absence of systematic screening, cases of congenital toxoplasmosis are probably underdiagnosed. Only the most severe forms, as shown by the recent retrospective survey of Pengsaa and Hattasingh (2015) in Thailand, are described. What is the true incidence of congenital infections, and what is the proportion of symptomatic forms and their clinical aspects remain to be determined. Regarding acquired toxoplasmosis in immunocompetent patients, our literature review did not find any description of severe forms as reported in South America. But this may be due to a lack of awareness of clinicians; toxoplasmosis is still not frequently evoked in case of pulmonary involvement or multiorgan failure. The presence of active ocular lesions in adults without retinal scars suggest the possibility of acquired cases of ocular toxoplasmosis, but its incidence is unknown. *Toxoplasma* infection is described in several Southeast Asian countries as the most frequent cause of infectious uveitis.

Chapter III: *Toxoplasma gondii* genetic diversity in Asia

Toxoplasma gondii has initially been described as having a clonal population structure comprising 3 predominant lineages (Types I, II and III) identified from 106 strains using PCR-RFLP. The description of this simple clonal structure resulted from a geographically limited sampling of strains from different hosts in North America and Europe (Howe and Sibley, 1995). More than 20 years later, due to genetic characterization of a larger number of strains originating from diverse continents, studied with a higher number of more discriminative genetic markers, the global population structure turned out to be much more complex, and closely related with geographical origin of the strains (Lehmann et al., 2006; Su et al., 2012).

I. *Toxoplasma gondii* genetic characterization methods and strain designation

Several molecular markers have been developed to study *T. gondii* genetic diversity. Microsatellite markers, PCR-RFLP and DNA sequencing typing are the techniques commonly used to determine the genotype.

I.1. Microsatellites

Microsatellite markers (MS) or STR (Short Tandem Repeats) are DNA fragments with variable number of short tandem nucleotide repeats (typically 1-6 nucleotides). The most frequently suggested polymorphism mechanism for microsatellites is strand slippage occurring during replication (Kelkar et al., 2008). Concerning *Toxoplasma gondii*, 15 microsatellite markers are currently used for genotyping (Ajzenberg et al., 2010). The 15 MS markers are located on 11 different chromosomes of *T. gondii* (Table 7). According to the level of polymorphism of these different markers, 2 levels of characterization may be obtained. The first level is a typing level which shows the ability of some markers to identify the clonal lineages and atypical strains. Eight microsatellite markers including *TUB2, W35, TgM-A, B18, B17, M33, IV.1* and *XI.1* were used as typing markers. The fingerprinting markers allow a second level of discrimination, with the ability to differentiate closely related strains within the same lineage. Seven microsatellite markers including *N60, N82, AA, N61, N83, M48* and *M102* were used as fingerprinting markers. Apart from the classical clonal lineages (type I, II, and III), strain clusters defined by genetic tools after MS genotyping are designated according to their prevalence in different geographical areas: *Africa 1, Africa 3*

(Ajzenberg et al., 2009; Mercier et al., 2010), *Amazonian*, *Caribbean 1*, *2*, *3* (Mercier et al., 2011), *Chinese 1* (Chen et al., 2011) etc... Genotypes that could not be clustered in these groups remain designated as "atypical" until further clustering. This technique shows a high resolution and is easy to perform (Ajzenberg et al., 2010). The high discriminatory power of MS genotyping is particularly useful for outbreak investigation, for analysing a laboratory contamination or detecting a mixed infection. Limitation of this technique is low DNA concentration, which induces an incomplete amplification. The threshold for genotyping has been established at about 33 ct obtained in qPCR. Another limitation is homoplasy (identical length of MS markers resulting from different mutation events during evolution), which may limit their use in the study of phylogenetic relationship (Estoup et al., 2002).

Table 7: Microsatellite markers currently in use for *T. gondii* genotyping.

Markers	Chromosome (position)	Size range (bp)	References
Typing markers			
TUB2	IX (974608 to 974896)	287-291	Ajzenberg et al., 2005
W35	II (633241 to 633482)	242-248	Ajzenberg et al., 2005
TgM-A	X (4824879 to 4825083)	203-211	Ajzenberg et al., 2005
B18	VIIa (2921536 to 2921693)	156-170	Ajzenberg et al., 2005
B17	XII (6474746 to 6475079)	334-366	Ajzenberg et al., 2005
M33	IV (672591 to 672760)	165-173	Blackston et al., 2001
IV.1	IV (742419 to 742693)	272-282	Ajzenberg et al., 2010
XI.1	XI (189702 to 190058)	354-362	Ajzenberg et al., 2010
Fingerprinting markers			
M48	Ia (332951 to 333166)	209-243	Blackston et al., 2001
M102	VIIa (3093491 to 3093664)	164-196	Blackston et al., 2001
N60	Ib (1766079 to 1766221)	132-157	Ajzenberg et al., 2002
N82	XII (1621472 to 1621585)	105-145	Ajzenberg et al., 2002
AA	VIII (5836880 to 5837144)	251-332	Ajzenberg et al., 2002
N61	VIIb (4217145 to 4217238)	79-123	Ajzenberg et al., 2002
N83	X (1772898 to 1773209)	306-338	Ajzenberg et al., 2002

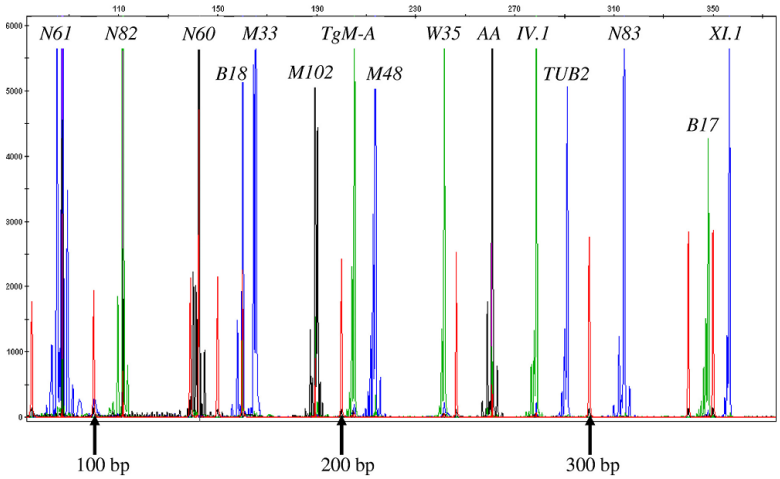


Figure 15: Electrophoresis of the 15-microsatellite markers multiplex PCR amplification, x axis showed a size fragment scale and y axis demonstrated a peak height scale (Ajzenberg et al., 2010).

I.2. Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP)

Single Nucleotide Polymorphisms (SNP) correspond to point mutations in the genome within a population. Single nucleotide base component of the DNA is modified. SNPs are very common throughout the genome. They occur within the coding and non-coding regions. Previously identified SNPs can be studied by PCR-RFLP (Restriction Fragment Length Polymorphism). Polymorphisms are detected by differences in restriction fragment sizes between individuals. The technique of PCR-RFLP is a widely used method for genotyping *T. gondii* isolates. The advantages of the common PCR-RFLP technique for *Toxoplasma* relies on nested PCR, which increases its sensitivity in case of with a small amount of DNA, but also can favor DNA contamination. Ten to eleven genetic markers including *SAG1, SAG2 (5'-3' SAG2 and alternative SAG2), SAG3, BTUB, GRA6, L358, c22-8, c29-2, PK1* and *Apico* are commonly analysed for genotyping. Each genotype, defined by the pattern of these 10-11 markers, is assigned a ToxoDB number (for Toxo DataBank number): ToxoDB#1, #2, #3… More than 200 ToxoDB genotypes are now recognized. The PCR-RFLP patterns are available at ToxoDB website (http://toxodb.org). This method is considered to be simple, sensitive, reproducible and cost-effective but agarose gels may be difficult to read (Su et al., 2010). Its main limitation is that it recognized only previously identified SNPs and may miss non identified SNPs in the target gene.

I.3. Multilocus Sequence Typing (MLST)

Multilocus sequence typing is a molecular technique using a number of housekeeping genes (loci). This technique has been used successfully to study the genetic population with a high resolution (Weiss and Kim, 2014). Genetic tools based on multilocus sequence typing cluster *T. gondii* strains into haplogroups. Firstly, a total of 46 strains were identified using the intron sequencing. The frequency of SNPs within 8 introns of 5 unlinked loci was analyzed. Eleven separate haplogroups were firstly identified (haplogroups 1 to 11). The results showed that the haplogroups 1, 2, 3 occurred mostly in North America and Europe while the haplogroups 4, 5, 8 and 10 occurred in South America and the haplogroup 6 was widespread (Khan et al., 2007). In 2011, Khan et al. analyzing 66 strains with the same markers, found another haplogroup, namely haplogroup 12, clustering strains from wild animal in North America (Khan et al., 2011). The haplogroups 1, 2, 3, 11 and 12 were common to North America and Europe, whereas the haplogroups 4, 5, 6, 8, 9 and 10 were common in South America (Khan et al., 2011). The haplogroups 13 and 14 were additionally identified by sequencing 8 introns from 5 unlinked genes when analyzing a new sampling of 74 strains including strains not only from North America, South America, Europe, but also from Africa and China. The Chinese isolates allowed to identify the haplogroup 13 and the African isolates revealed the haplogroup 14 (Khan et al., 2011). In 2012, through a collaborative work, 956 strains originating from diverse continents were studied by 15 MS markers, 12 PCR-RFLP markers, and sequencing of 4 introns from 3 genes (*UPRT*, *EF*, *HP*) (Su et al., 2012). Clustering methods applied to PCR-RFLP results allow to select 138 representative strains among the 956 strains initially studied. These 138 representative strains were further analyzed by intron sequencing and MS markers. Fifteen haplogroups were identified by using the 4 intron sequences from 138 RFLP representative strains (figure 16). The Bayesian clustering method called STRUCTURE was used to group the haplogroups into ancestral clades. The system of clades organized the haplogroups into 6 major clades; clade A comprised haplogroups 1, 6 and 14; clade B included haplogroups 4 and 8; clade C contained haplogroup 3; clade D defined haplogroups 2 and 12; clade E comprised haplogroup 9 and clade F defined haplogroups 5, 10 and 15 (Su et al., 2012).

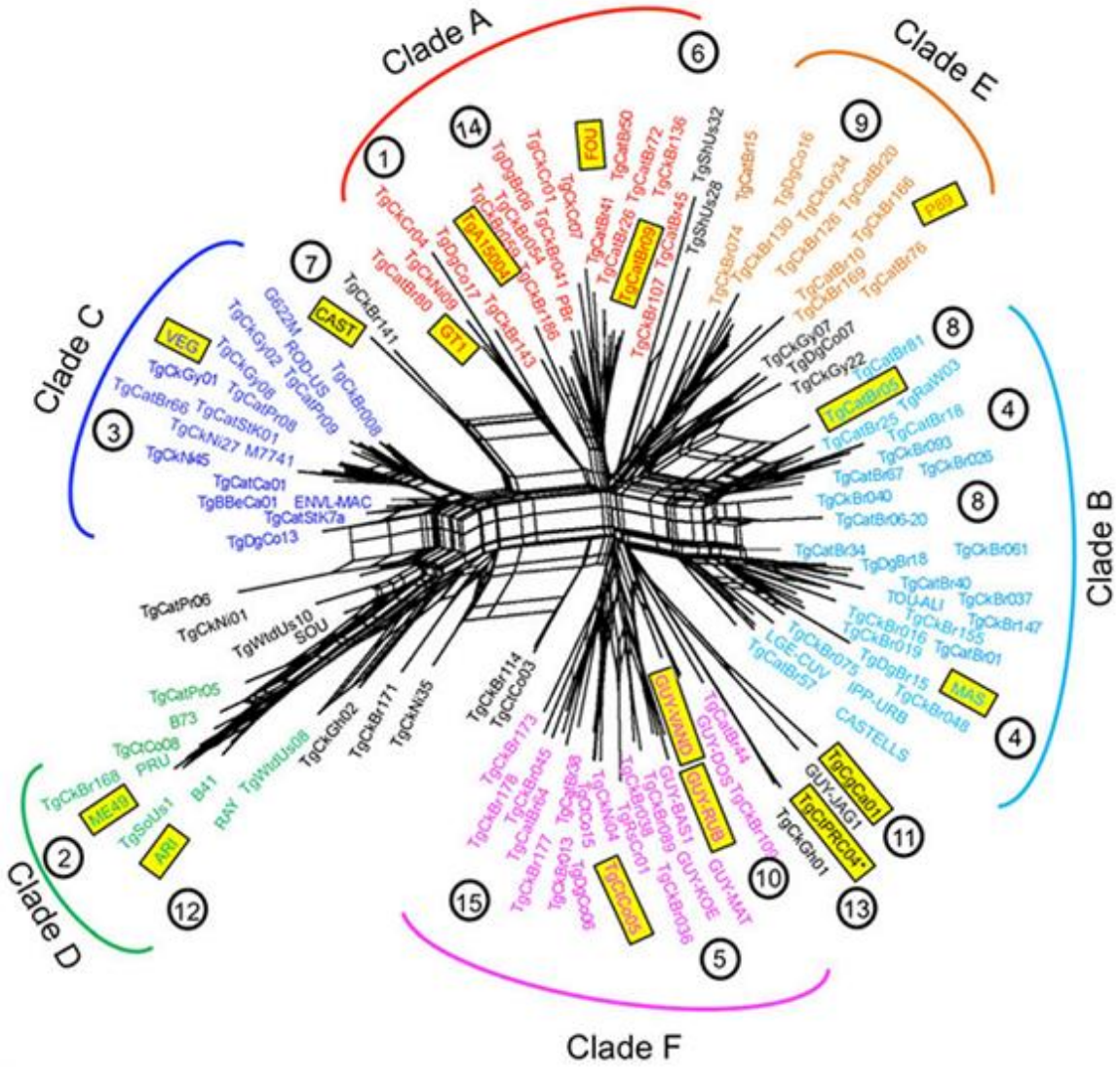


Figure 16: 138 isolates of *T. gondii* classified into 16 haplogroups (combination of data from 12 PCR-RFLP markers and sequencing of 4 introns from 3 genes) (Su et al., 2012).

The last technique used for genetic characterization is whole genome sequencing. The whole genome sequences from 62 representative isolates were used to characterize the genetic variation of *T. gondii* and the analysis showed the 16 major haplogroups, clustered into 6 ancestral clades (Figure 17) (Lorenzi et al., 2016). Whole genome sequencing captures the totality of genetic polymorphism, allowing considering that this last picture of global diversity of *Toxoplasma* is the more robust. Actually, some differences are observed when comparing clusters obtained with the combination of PCR-RFLP markers and sequencing of 4 introns (Su et al., 2012) and whole genome sequencing (Lorenzi et al., 2016); among them, the individualization of haplogroup 16 previously included in haplogroup 4, or the absence of clear clustering of haplogroups 5 and 10 (*Amazonian* strains as defined by MS markers). Ancestral clades defined by whole genome sequencing also differed from (Su et al., 2012).
However, this last picture should not be considered as a definitive one as it concerns only 62 representative strains. Strains originating from Africa or Asia are underrepresented in this panel.

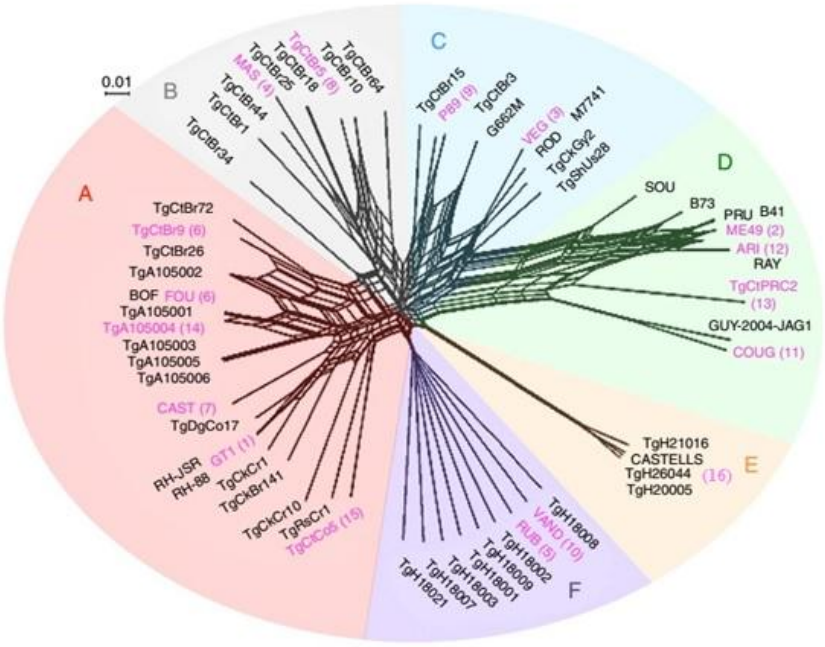


Figure 17: 62 isolates of *T. gondii* classified into 6 ancestral groups and 16 haplogroups (data from whole genome sequencing) (Modified from Lorenzi et al., 2016).

II. Geographical distribution of *Toxoplasma gondii* genotypes in Asia: a link with neighboring continents. (Submitted review)

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Abstract

Defining the pattern of genetic diversity of *Toxoplasma gondii* is important to understand its worldwide distribution. During the last decades, a large number of studies have been published on *Toxoplasma* genotypes circulating in Europe, in North and South America. Two continents are still largely unexplored, Africa and, to a less extent, Asia. In this last continent, an increasing number of publications reported genotypes circulating in diverse provinces of China, but very few data are available for other Asian countries. After a systematic database search, 47 papers related to *T. gondii* genotypes in Asia were analyzed. Genetic characterization of DNA was performed by microsatellite markers, or more usually by a multiplex PCR using 11 PCR-RFLP markers, allowing data comparison to draw a first global picture of the population structure of this parasite throughout Asia. Overall, 418 isolates or DNA extracts were completely typed by PCR-RFLP or microsatellite marker methods, revealing 36 different PCR-RFLP or equivalent microsatellite genotypes: 15 genotypes identified by a ToxoDB number and 21 atypical or unique genotypes. The most common genotype found in Asia is the genotype ToxoDB#9 (*Chinese 1*). The clonal types I, II and II variant, and III were also commonly found in Asia. The geographical distribution of these genotypes across Asia may reflect either a continuum with Europe for the western part of Asia (presence of type II), or the circulation of strains through animal migration or human activities between Africa and the Southwestern part of Asia (*Africa 1* genotype in Turkey). Although there are some indications of a genetic population structure in Southeast Asian countries different from the rest of Asia, more studies in this tropical part of Asia will be necessary for a region which represent as well as Africa one of the missing links of the *T. gondii* genetic diversity.

Key words: *Toxoplasma gondii*, Asia, genetic diversity, rodent migration, PCR-RFLP, Microsatellite markers.

1. Introduction

Toxoplasma gondii is one of the most common parasites, infecting human and other warm-blooded animals. It is estimated that *T. gondii* infects one third of the world population (Weiss and Dubey, 2009). Human infection rates vary around the world depending on several sociogeographical factors. Acquisition of *T. gondii* infection is commonly acquired by the ingestion of tissue cysts that contain bradyzoites or by the ingestion of oocysts containing sporozoites. The sources of human infection could vary greatly between different ethnic groups and different geographical locations.

Toxoplasma gondii strains have now been isolated and identified in a large number of hosts (humans and wild and domestic animals). Several molecular markers have been developed to evaluate *T. gondii* genetic diversity. According to the techniques used for characterizing *Toxoplasma* strains, different designations may be encountered. Correspondence between these designations is presented in Table 1. The last global population structure picture based on DNA sequence typing and whole genome sequencing of 62 representative strains identified 16 haplogroups originating from 6 ancestral populations or clades (Su et al., 2012; Lorenzi et al., 2016). They clustered genotypes defined by multilocus markers, either a set of 11 PCR-RFLP genetic markers (ToxoDB#1, #2, #3 ...) (Su et al., 2006, 2010, Toxodb website (http://toxodb.org/toxo/) or a set of 15 microsatellite (MS) markers (Ajzenberg et al., 2010).

Table 1: Distribution of *Toxoplasma gondii* genotypes defined by 11 PCR-RFLP or 15 MS markers in the different haplogroups and ancestral populations.

Whole genome sequencing (clades) Lorenzi et al., 2016	Sequence typing (Haplogroup) Su et al., 2012; Lorenzi et al., 2016	PCR-RFLP (ToxoDB #) www.toxodb.org; Su et al., 2012; Dardé et al., 2014	Microsatellites (Type or geographical designation)[a] Ajzenberg et al., 2010; Su et al., 2012 (Supporting Information)
A	1	**10 (type I)**, 27, 35, 38, 55	Type I
	6	**6**, 86 33, 41, 42, 51, 56,70, 80, 82, 84, 85, 105, 117	*Africa 1*
	7	**28,** 77	Atypical
	14	**203** 36, 88	*Africa 3*
	15	23, 44, 61, 63, 81, 101, 109, **111**, 134, 135, 136	Atypical
B	4	**11**, 17, 47, 76, 92, 93, 99, 104, 106, 107, 108, 119, 124, 126, 202	Atypical
	8	**19**, 32, 40, 53, 59, 64, 69, 71, 75, 94, 121	Atypical
C	3	**2 (type III)**, 7, 50, 72, 133, 140 13 12, 31 141 25, 26, 79, 83, 90, 115, 118, 125, 130,	Type III *Caribbean 1* *Caribbean 2* *Caribbean 3*

	9	**8**, **14,** 21, 45, 46, 67, 78, 114, 116, 120, 123, 138	Atypical
D	2	**<u>1 (type II)</u>**, **<u>3 (type II variant)</u>**, 128 127, 129	Type II
	11	**66**, 197	Atypical
	12	**4, 5**, 39, 49, 74	Atypical
	13	**<u>9</u>** 137	*Chinese 1*
E	16	15	Atypical HG16
F	5	**60**[b], 95, 98, 100 22, 37, 52, 65,	*Amazonian*
	10	**60**[b], 97 34, 96	*Amazonian*

[a.] MS genotypes corresponding to PCR-RFLP genotypes on the same line in the table

[b.] ToxoDB #60 was found by Su C. et al, 2012 within both Haplogroup 5 and 10

*The underlined genotype numbers are the genotypes found in Asia which have been included in a given haplogroup.

** The bold genotypes correspond to the most frequent genotypes of the haplogroup.

The global distribution of *T. gondii* genotypes is widely known in Europe, North and South America. The clonal lineages Type II and, to a less extent, type III predominate in Europe (Dardé et al., 2014). They are also present in North America, but a fourth clonal lineage (haplogroup 12) and other atypical genotypes are prevalent in this part of the world (Dubey et al., 2011). A high diversity was found in South America with no predominant genotype (Shwab et al., 2014). The diversity on the African continent is less well known: apart from type II and III, other genotypes were identified by MS markers as *Africa 1* (belonging to haplogroup 6) that circulates in large area of Western and Central Africa and *Africa 3* in Gabon (Mercier et al., 2010), or by PCR-RFLP markers such as the ToxoDB#20 genotype found in Egypt (Al-Kappany et al., 2010) and Ethiopia (Dubey et al., 2013).

In Asia, an increasing number of studies have been conducted on strains circulating in China. A largely predominant genotype, *Chinese 1* (ToxoBD#9) is found in diverse provinces of China. But little is known about strains circulating in other Asian countries. This study aims through a literature review on genetic diversity of *T. gondii* in Asia to understanding the circulation and propagation of *T. gondii* strains in Asia, and to propose hypotheses about their relations with strains circulating on neighboring continents.

2. Materials and methods

2.1 Literature search

To identify published studies on genetic characterization of *T. gondii* strains in Asia, we conducted a systematic search of the literature published in English until September 2016. We used online databases MEDLINE and Google Scholar to find the information. The keywords and research equations used were: *Toxoplasma gondii* [AND] genotypes [AND] Asia, and *Toxoplasma gondii* [AND] genotypes [AND] each country in Asia (Figure 1).

Box 1: list of Asian countries selected for literature search.

Afghanistan, Armenia, Azerbaijan, Bahrain, Bangladesh, Bhutan, Brunei, Cambodia, China, Cyprus, Georgia, India, Indonesia, Iran, Iraq, Israel, Japan, Jordan, Kazakhstan, Korea, Kuwait, Kyrgyzstan, Laos, Lebanon, Malaysia, Maldives, Mongolia, Myanmar, Nepal, Oman, Pakistan, Palestine, Philippines, Qatar, Russia, Saudi Arabia, Singapore, Sri Lanka, Syria, Taiwan, Tajikistan, Thailand, Timor-Leste, Turkey, Turkmenistan, United Arab Emirates, Uzbekistan, Vietnam, Yemen

* Underlined countries correspond to the countries where *T. gondii* strains were genotyped.

2.2 Selection of studies

Articles were selected based on their titles and then their abstracts. Only those articles that met the exclusion criteria were finally included. Those retained were read in full. Exclusion criteria were: (i) publications that do not describe *T. gondii* genotypes in Asia, (ii) publications using genetic characterization methods with less than 5 typing markers, considering that, they do not allow a reliable genotyping (Fig. 1). Some papers presenting incomplete genotypes were considered for discussion, notably for countries where very few genotyping data were available. For publications before 2010 using PCR-RFLP markers, the genotype number was not defined, and the database ToxoDB (http://toxodb.org/toxo/) was used to attribute a ToxoDB genotype number according to the combination of alleles. It was also used to retrieve geographical distribution of a given genotype outside Asia. The results of literature review were classified by country.

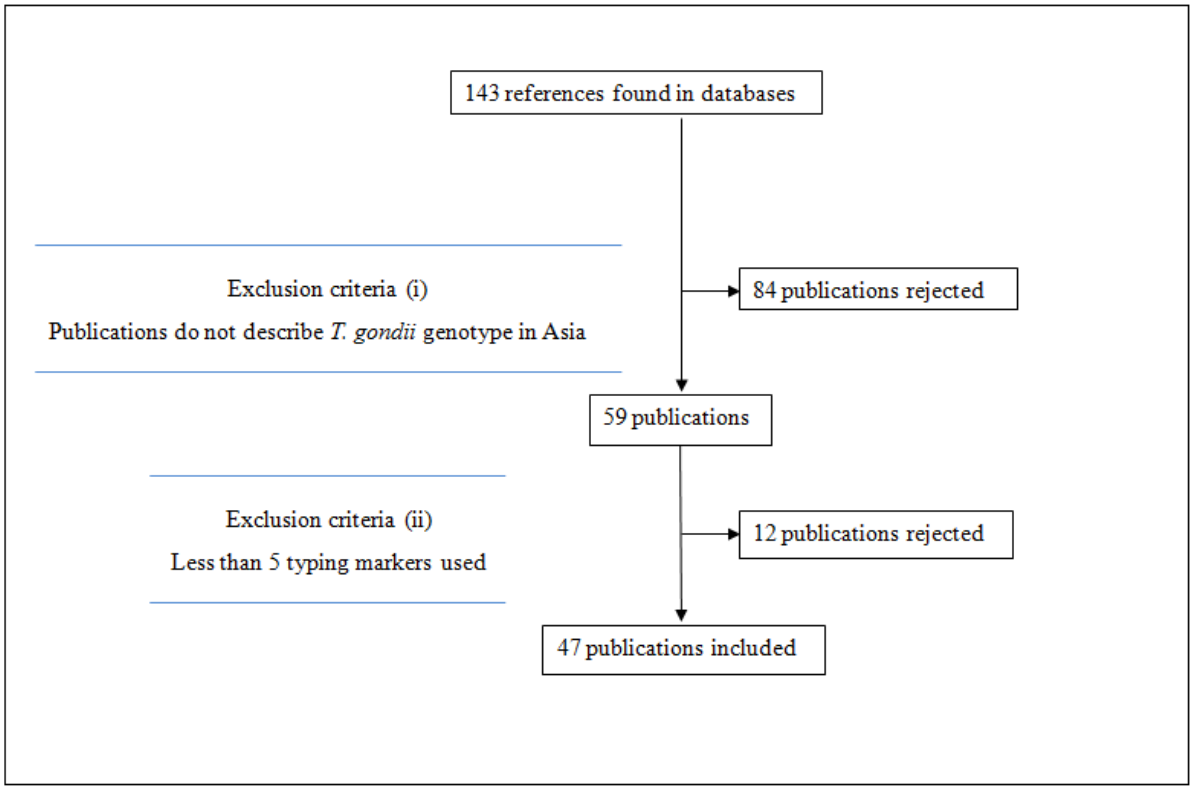

Figure 1: Flow chart showing the selection procedure for the publications.

3. Results

In Asia, a total of 442 samples were genotyped with a method using at least 5 markers. Only 4 studies used less than 9 markers: two from Malaysia (15 isolates genotyped by 7 PCR-RFLP markers) (Puvanesuaran et al., 2013a, 2013b), one from Shangai (1 isolate typed by 5 PCR-RFLP markers and UPRT-1 sequencing) (Zhou et al., 2013), and one from Iran (16 isolates typed with 5 MS markers and GRA6 sequencing) (Zia-Ali et al., 2006). They were considered for building the global pattern of *T. gondii* genotypes in Asia. Among the studies using 9-11 PCR-RFLP markers, 24 isolates or DNAs were not fully genotyped and the authors themselves considered that the ToxoDB genotype could not be determined. So, 418 samples were considered for our analysis (Table 2). These 418 samples revealed 36 different PCR-RFLP genotypes (15 genotypes identified by a ToxoDB number and 21 atypical or unique genotypes) (Table 2). For the studies using MS markers or other markers, results were expressed as type lineages or, when possible, as equivalent ToxoDB genotype. The most common genotype found in Asia was the genotype ToxoDB#9 (*Chinese* 1). This genotype was mostly found in China and, to a less extent in Sri Lanka and Vietnam. The genotypes ToxoDB#1 and #3 (Type II and II variant), #2 (Type III), and #10 (Type I) were also commonly found in Asia. The majority of genotyped strains were from China (306/418, 73.2%). The remaining 112 strains were distributed in the following countries: 24/418 (5.7%) in Sri Lanka, 24/418 (5.7%) in Turkey, 19/418 (4.5%) in Myanmar, 16/418 (3.8%) in Iran, 15/418 (3.6%) in Malaysia, 8/418 (1.9%) in Vietnam, 3/418 (0.7%) in United Arab Emirates, 1/418 (0.2%) in Indonesia, 1/418 (0.2%) in Korea, and 1/418 (0.2%) in Qatar.

Table 2: Different isolates or DNAs of *T. gondii* from Asia with a complete PCR RFLP or MS genotypes.

Countries	Samples	Sample size (isolate/DNA)	Techniques	Genotypes (#ToxoDB number)	References
China North					
Beijing	Stray cats	11 isolates	PCR RFLP 11 markers	11 #9	Qian et al., 2012
Shanxi	Cats	2 isolates	15 MS markers	2 Chinese 1 (eq. #9)*	Li et al., 2014
	Cats	2 isolates	PCR RFLP 9 markers	2 #9	Chen et al., 2011
China Northeast					
Jilin	Free living *Microtus fortis* (reed vole)	8 DNAs	PCR RFLP 10 markers	4 #9 4 #10	Zhang et al., 2014
	Bat	3 DNAs	PCR RFLP 11 markers	2 #9 1 #10	Qin et al., 2014
	Arctic foxes (*Vulpes lagopus*)	2 DNAs	PCR RFLP 11 markers	2 #10	Zhang et al., 2016
	Wild waterfowls	2 DNAs	PCR RFLP 11 markers	2 #9	Zhang et al., 2015
	Cattle	1 DNAs	PCR RFLP 11 markers	1 #10	Ge et al., 2014
	Sika deer	6 DNAs	PCR RFLP 11 markers	6 #9	Cong et al., 2016
Liaoning	Pigs	15 isolates	PCR RFLP 10 markers	13 #9 2 #3	Wang et al., 2016
Heilongjiang	Artic foxes (*Vulpes lagopus*)	2 DNAs	PCR RFLP 11 markers	2 #9	Zhang et al., 2016
China Northwest					
Gansu	White yaks	2 DNAs	PCR RFLP 11 markers	2 #9	Qin et al., 2015
	Ground-tit (house sparrow)	4 DNAs	PCR RFLP 9 markers	4 #3	Cong et al., 2013
	Pet birds	4 DNAs	PCR RFLP 10 markers	4 #3	Cong et al., 2014
	Pig	1 isolate	PCR RFLP 10 markers	1 #9	Zhou et al., 2009
Qinghai	Sheep	1 isolate	PCR RFLP 10 markers	1 #3	Zhou et al., 2009
	Qinghai vole (*Microtus fuscus*) Plateau pika (*Ochotona curzoniae*) Tibetan ground-tit (*Pseudopodoces hulilis*)	6 DNAs	PCR RFLP 10 markers	4 #10 2 new genotypes	Zhang et al., 2013
China West					
Xinjiang	Wild birds	2 DNAs	PCR RFLP 10 markers	2 #3	Huang et al., 2012
China Southwest					
Guizhou	Pigs, dogs, cats	5 isolates	PCR RFLP 11 markers	5 #9	Li et al., 2015
	Cats Pigs	5 isolates 4 isolates	PCR RFLP 11 markers	5 #9 4 #9	Wang et al., 2013b
	Cat	1 isolate	15 MS markers	1 Chinese 1 (eq.#9)*	Li et al., 2014
Yunnan	Black goats	8 DNAs	PCR RFLP 11 markers	1 #9 7 #10	Miao et al., 2015
	Bats	2 DNAs	PCR RFLP 11 markers	2 #10	Jiang et al., 2014
	Cats	16 DNAs	PCR RFLP 11 markers	1 #1 1 #3	Tian et al., 2014

				11 #9 1 #20 2 #225	
Guangxi	Bats	3 DNAs	PCR RFLP 11 markers	1 #9 2 #10	Jiang et al., 2014
Sichuan	Pigs	3 DNAs	PCR RFLP 11 markers	3 #9	Jiang et al., 2013
Chongqing	Pig	1 DNA	PCR RFLP 11 markers	1 #9	Jiang et al., 2013
China Center					
Henan	Cats	9 isolates	PCR RFLP 10 markers	1 #1 1 #2 6 #9 1 #17	Yang et al., 2015
	Giant panda	1 DNA	PCR RFLP 10 markers	1 atypical genotype	Ma et al., 2015
	Pet dogs	2 DNAs	PCR RFLP 11 markers	1 new genotype	Qian et al., 2015
	Pigs	13 DNAs	PCR RFLP 10 markers	6 #9 7 #10	Zhou et al., 2010
Anhui	Humans, cancer patients	9 DNAs	PCR RFLP 10 markers	9 #9	Wang et al., 2015
	Humans, cancer patients	2 isolates	PCR RFLP 10 markers	1 #1 1 #204	Wang et al., 2013a
	Chicken	1 isolate	PCR RFLP 11 markers	1 #225	Wang et al., 2013b
	Cats Pork	2 isolates 1 isolate	15 MS markers	2 Chinese 1 (eq. #9) 1 Chinese 1 (eq. #9)	Li et al., 2014
	Cats	2 isolates	PCR RFLP 9 markers	2 #9	Chen et al., 2011
	Pigs	2 DNAs, 1 isolate	PCR RFLP 9 markers	1 #9 2 #213	Wang et al., 2012
Hubei	Cats	6 isolates	PCR RFLP 10 markers	6 #9	Wang et al., 2013a
	Vole	1 isolate	PCR RFLP 11 markers	1 #9	Wang et al., 2013b
	Cats	12 isolates	15 MS markers	12 Chinese 1 (eq. #9)	Li et al., 2014
	Cats	8 isolates	PCR RFLP 9 markers	6 #9 2 # Chinese 2	Chen et al., 2011
	Pigs	3 DNAs	PCR RFLP 10 markers	3 #10	Zhou et al., 2010
China South					
Hainan	Geese	2 DNAs	PCR RFLP 10 markers	2 #1	Rong et al., 2014
Hunan	Pig	1 isolate	PCR RFLP 10 markers	1 #10	Zhou et al., 2009
Guangdong	Cats	17 isolates	PCR RFLP 11 markers	15 #9 2 #18	Dubey et al., 2007c
	Cats Pig Human	8 isolates 2 isolates 1 isolate	PCR RFLP 10 markers	8 #9 2 #9 1 #9	Zhou et al., 2009
	Bat	2 DNAs	PCR RFLP 11 markers	2 #10	Qin et al., 2014
	Pig	1 DNA	PCR RFLP 11 markers	1 #3	Jiang et al., 2013
	Humans, AIDS patients	2 isolates	PCR RFLP 10 markers	1 #9 1#10	Wang et al., 2013a
	Cats	2 isolates	15 MS markers	2 Chinese 1 (eq.#9)	Li et al., 2014
	Cats	2 isolates	PCR RFLP 9 markers	2 #9	Chen et al., 2011
China East					
Shanghai	Rabbits	1 isolate	PCR RFLP 5	1 Type III	Zhou et al., 2013

			markers + UPRT-1 sequencing		
	Human	1 isolate	PCR RFLP 10 markers	1 #10	Zhou et al., 2009
Jiangsu	Rodents	4 DNAs	PCR RFLP 10 markers	4 #9	Yan et al., 2014
	Mice	3 DNAs		3 #9	
	Pig	1 isolate	PCR RFLP 10 markers	1 #10	Zhou et al., 2009
	Cats	8 isolates	PCR RFLP 10 markers	3 #9 1 #10 4 #205	Wang et al., 2013a
	Cats	8 isolates	15 MS markers	3 Chinese1 (eq.#9) 1 Type 1 4 eq.# 205	Li et al., 2014
Jiangxi	Bat	3 DNAs	PCR RFLP 11 markers	3 #9	Qin et al., 2014
	Pigs	12 DNAs	PCR RFLP 11 markers	12 #9	Jiang et al., 2013
Shangdong	Human, cancer patients	17 DNAs	PCR RFLP 11 markers	8 #9 9 #10	Cong et al., 2015
	Cats	5 isolates	PCR RFLP 10 markers	5 #9	Wang et al., 2013a
	Artic foxes (*Vulpes lagopus*)	1 DNA	PCR RFLP 11 markers	1 #9	Zhang et al., 2016
Fujian	Zoo wildlife and Pet birds	3 DNAs	PCR RFLP 12 markers	1 #2 1 #9 1 #10	Chen et al., 2015
	Wild bird	1 DNA	PCR RFLP 10 markers	1 #10	Huang et al., 2012
Zhejiang	Human	1 isolate	PCR RFLP 10 markers	1 atypical genotype	Zhou et al., 2009
South Korea					
	Human, ocular lesion	1 isolate	PCR RFLP 9 markers	1 #10	Quan et al., 2008
Indonesia					
12 towns (not specified)	Free-range chickens	1 isolate	PCR RFLP 10 markers	1 #89	Dubey et al., 2008
Malaysia					
Peninsular Forests of Pahang	Wild boars	11 isolates	PCR RFLP 7 markers	11 Type I	Puvanesuaran et al., 2013a
Peninsular	Free-range ducks	4 isolates	PCR RFLP 7 markers	4 Type I	Puvanesuaran et al., 2013b
Myanmar					
South, Sedon and Wutao counties	Bats	19 DNAs	PCR RFLP 11 markers	4 #10 15 atypical genotypes	Sun et al., 2013
Vietnam					
7 provinces, Binh Phuoc, Binh Duong, Dong Nai, Tay Ninh, Tien Giang, Ben Tre and Long An	Unwanted dogs	8 isolates	PCR RFLP 11 markers	4 #9 4 #18	Dubey et al., 2007a
Sri lanka					
	Street dogs	24 isolates	PCR RFLP 11 markers	3 #2 1 #9 9 #20 11 atypical (identical genotype)	Dubey et al., 2007b
Iran					
Tehran and Mazandaran	Ducks	1 isolate	5 MS markers GRA6	1 Type III	Zia-Ali et al., 2007
	Stray cats	2 isolates		2 Type II	
	Humans	3 isolates		2 Type II	

			sequencing	1 Type III	
	Sheep	4 isolates		2 Type II 2 Type III	
	Free-range chickens	6 isolates		6 Type III	
Turkey					
Ankara, Balikesir Anatolia	Newborns	2 isolates	15 MS markers	2 *Africa* 1	Döşkaya et al., 2013
Izmir	Stray cats	22 isolates	15 MS markers	2 Type III 19 Type II 1 *Africa* 1	Can et al., 2014
Qatar					
	Sand cats	1 isolate	PCR RFLP 11 markers	1 #3	Dubey et al., 2010
United Arab Emirates					
	Sand cats	3 isolates	PCR RFLP 11 markers	2 #20 1 atypical	Dubey et al., 2010

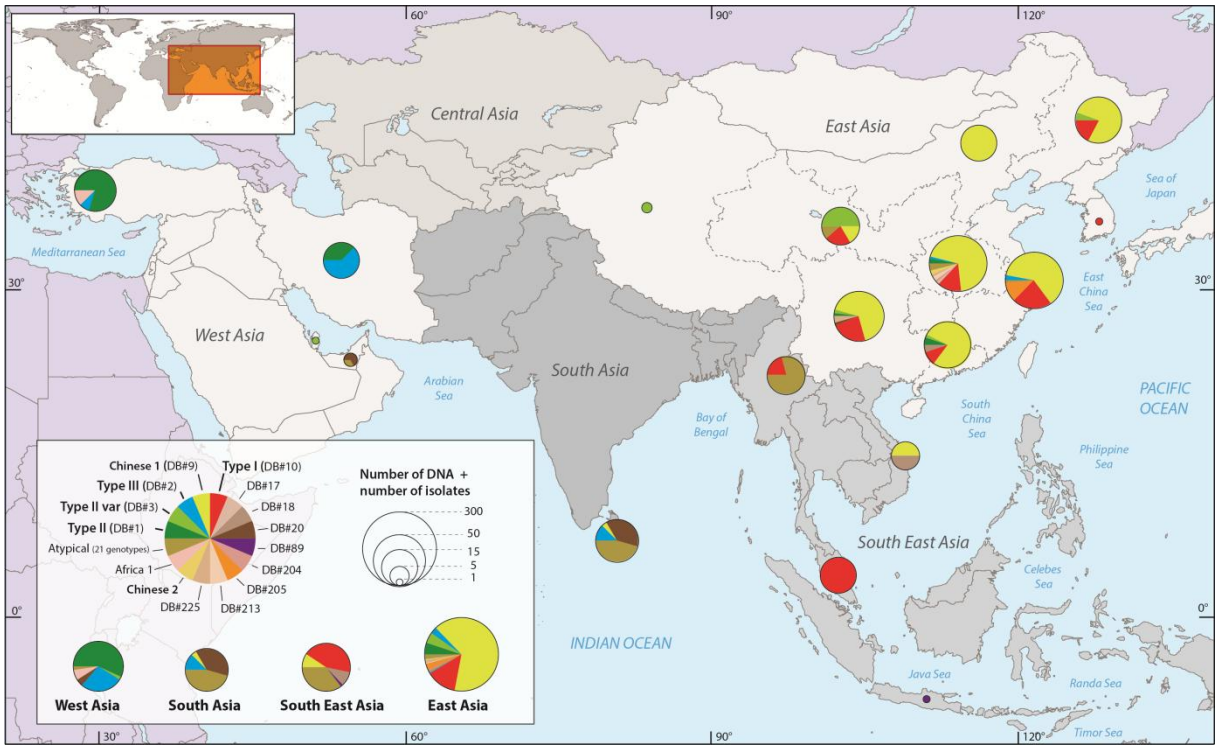

Figure 2: Geographical distribution of *T. gondii* strains in Asia.

3.1 Genetic diversity in Central and Eastern Asia

3.1.1 China

We decided to present *T. gondii* genotypes found in China per region, North, Northeast, Northwest, South, Southwest, East, West and Center of China (Fig. 2). Currently, a total of 306 samples (154 DNA extracts and 152 isolates) were characterized. The samples included 33 human and 273 animal strains (220 from domestic animals and 53 from wild animals). Thirteen ToxoDB genotypes and 5 atypical genotypes were identified in China. The most common genotype was ToxoDB#9 (*Chinese 1*) with 205/306 strains (66.9% of total samples). It is largely distributed over China, but mainly found in the Northern, Southern and Center parts of the country. ToxoDB#10 (Type I) is the second most common genotype found in China with 52 strains over 306 (16.9%),

mainly in the eastern and southwestern provinces of China, respectively 15/69 (21.7%) and 11/48 (22.9%) strains of these regions. The clonal types II (ToxoDB#1 and #3), and III (ToxoDB#2), and the genotype #205 were less frequently found. Altogether, Type II (5 ToxoDB#1) and II variant (15 ToxoDB#3) represented 6.5% of the genotypes (20/306). Thirteen of them (65%) were found in the western (Xinjiang), northwestern (Gansu and Qinghai) and central (Henan and Anhui) provinces of China. The 7 remaining samples are occasionally found in Southwest (2 in cats from Yunnan), South (2 in Hainan geese and 1 in a pig from Guangdong), and Northeast (2 in Liaoning pigs). Only 3 samples were typed as Type III or ToxoDB#2, 1 in Henan, a province from central China, and 2 in the eastern parts of the country (1 in a Shanghai rabbit, 1 in Fujian). Eight samples from Jiangsu province in the eastern part of the country gave genotype ToxoDB#205. Other genotypes are infrequently encountered in China (15/306): #17 (1 sample in Henan), #18 (2 samples in Guangdong), #20 (1 sample in Yunnan), #204 (1 in Anhui), #213 (2 in Anhui), #225 (1 in Anhui) and 7 atypical strains not referenced in ToxoDB.

3.1.2 South Korea

One type I isolate named KI-1 was isolated from a patient with ocular lesions (Quan et al., 2008; Lin et al., 2005). Two other studies were conducted on *Toxoplasma* genotyping in this country. Although they were not retained for this analysis because relying on an insufficient number of markers, they also showed type I alleles for DNA samples of stray-cats (Kim et al., 2009) and rodents (Hong et al., 2014).

3.1.3 Japan (not presented in Table 2)

There is no publication using more than 5 genetic markers for *Toxoplasma* genotyping in Japan. Two studies based on GRA6 PCR-RFLP revealed either type I, II or III alleles for this gene, but GRA6 sequencing showed a higher polymorphism (Zakimi et al., 2006; Kyan et al., 2012).

3.2 Genetic diversity in Southeast Asia

3.2.1 Indonesia

One isolate from free-range chicken gave the genotype ToxoDB#89 (Dubey et al., 2008). This genotype ToxoDB#89 was found only in Indonesia.

3.2.2 Malaysia

Of the 19 isolates obtained in Peninsular Malaysia from 4 free-range ducks (2 isolates per duck) and 11 wild boars, 15 samples were genotyped as Type I with 7 PCR-RFLP markers. Four samples had incomplete typing, 2 with Type II alleles and 2 with Type I alleles (Puvanesuaran et al., 2013a, 2013b). The 11 wild boars that were all infected by *T. gondii* type I were living in rainforests of Pahang which host a variety of wild felids. The 4 other Type I were found in free-range ducks from the district of Kulim in Kedah state, close to the Thai border. This suggests that type I may be widely distributed in this country in wild animals as well as in domestic ones.

3.2.3 Myanmar

Nineteen DNA extracts were collected from bats in Sedon and Wutao counties, Myanmar, close to Yunnan province, China. Four extracts belonged to genotype ToxoDB#10 and the 15 remaining samples gave 14 different atypical genotypes with a majority of type I alleles revealing a high diversity among these wild animals (Sun et al., 2013).

3.2.4 Vietnam

A total of 8 strains isolated from unwanted dogs were characterized. Two genotypes were found: 4 ToxoDB#9 and 4 ToxoDB#18 genotypes (Dubey et al., 2007a). This last genotype was also described in the nearby province of Guandgong in South China (Dubey et al., 2007c).

Overall, for these tropical Southeast Asian countries, only 43 *Toxoplasma* strains were completely characterized, most of them (34/43) were type I strains or atypical genotypes with a majority of Type I alleles. They were mainly found in wild animals (wild boars in Malaysia, bats in Myanmar). The presence of the ToxoDB#9 genotype in Vietnam confirms its large distribution in Asia.

3.3 Genetic diversity in Western Asia

3.3.1 Sri Lanka

A total of 24 isolates from street dogs were characterized. A variety of genotypes was found in this relatively small number of samples, 3 corresponded to genotype ToxoDB#2 (type III), 1 to ToxoDB#9, 9 to ToxoDB#20 and 11 presented a single atypical genotype, not referenced in ToxoDB. (Dubey et al., 2007b).

3.3.2 India (not presented in Table 2)

On the large neighboring Indian subcontinent, a recent genotyping study based on PCR-RFLP and sequencing of 4 markers (βTUB, GRA6, alternative SAG2 and SAG3) on DNA from tissues obtained at postmortem in AIDS patients suggested that majority of the patients (22/25; 88%) in South India are infected with strains that are recombinants of type II/III and/or strains different from the archetypal lineages I, II, and III (Vijaykumar et al., 2016).

3.3.3 Iran

Six isolates from Tehran and Mazandaran, Iran were characterized as type II and 10 isolates as genotype Type III. These isolates derived from ducks, stray cats, sheep, free-range chickens and humans (Zia-Ali et al., 2007).

3.3.4 Turkey

In Izmir, Turkey, 22 isolates from stray cats were characterized using 15 MS markers. Nineteen samples were identified as Type II genotype, 2 as Type III genotype, 1 as *Africa* 1 genotype (HG 6) (Can et al., 2014). This last genotype, *Africa* 1, was also present in two strains from Anatolia, named Ankara and Ege-1 isolated from newborns with congenital toxoplasmosis. Ankara and Ege-1 strain killed mice in 4-5 days (Döşkaya et al., 2013).

3.3.5 Qatar

Only one sample (ToxoDB#3 genotype) was recovered from sand cats in Qatar (Dubey et al., 2010).

3.3.6 United Arab Emirates

Of the three samples from sand cats that were collected and typed, 2 belonged to genotype ToxoDB#20 and 1 had an incomplete atypical genotype (Dubey et al., 2010).

3.4 Genotypes shared between Asia and other continents

To appreciate the possible circulation of genotypes between Asia and other continents, we focused on the distribution of genotypes found in Asia in other locations. This search was mainly based on data obtained from the toxodb website and it may not be exhaustive. Isolates from human origin were excluded as their geographical origin could not be ascertained.

Table 3: Sharing of *T. gondii* genotypes between Asia and other locations.

Genotypes	Number in Asia	Locations in Asia	Other locations (www.toxodb.org)
#1 and #3 (Type II lineage)	30	China, Iran, Turkey, Qatar	Europe, Africa: Senegal, Egypt, Mali, Kenya Americas: USA, Brazil, Chile, Costa Rica, Saint Kitts, Australia
#2 (Type III)	18	China, Iran, Sri Lanka, Turkey	Europe: mostly South Europe, North America: USA, Africa: Egypt, Burkina Faso, Congo, Mali, South and Central America: Brazil, Argentina, Chile, Mexico, Peru, Guyana, Panama, Saint Kitts, Costa Rica, Grenada, Nicaragua,
#9 (*Chinese 1*)	210	China, Sri Lanka, Vietnam	South America: Colombia, Mexico, North America: USA
#10 (Type 1)	72	China, Korea, Malaysia, Myanmar	South America: Brazil, Colombia, Uruguay, North America: USA
#17	1	China	South America: Argentina, Brazil, Peru
#18	6	China (Guangdong), Vietnam	Colombia
#20	12	China, United Arab Emirates, Sri Lanka	Egypt, Ethiopia
#89	1	Indonesia	-
#204	1	China	-
#205	8	China	-
#213	2	China	-
#225	3	China	-
Africa 1 (MS)	3	Turkey	Africa: Gabon, Cameroon, South America: Brazil (Br I)

4. Discussion

As expected, the major finding of this literature review is the overwhelming presence of ToxoDB#9, also designated as *Chinese 1*, throughout different provinces of China and its dispersion in other Asian countries, such as Vietnam and Sri Lanka. This large geographical distribution is a criterion for considering *Chinese 1* as a major clonal lineage together with the other clonal lineages such as type I, II or III. The emergence of type I, II or III clonal lineages has been reported to the apparition of agriculture and domestication of animals, 10,000 years ago in the Fertile Crescent of Near East (Khan et al., 2007), favoring the selection of a few number of strains well adapted to domestic animals. Similarly, domestication of cats and intermediate hosts is very ancient in China and could have led to a clonal expansion of *Chinese 1* (Li et al., 2014). Domestication of pigs and cattle

is reported to have occurred in China 9,000-10,000 years ago, that of sheep and goats about 3,600-3,000 BC in Gansu and Qinghai provinces (Jing, 2008). Up to now, the presence of domestic cats seems to have occurred later than in the Near East. Archeological findings dated it back to 5300 years ago in an early agricultural village in Shaanxi, China (Hu et al., 2014).

Sequencing of introns recognized *Chinese 1* genotype as belonging to haplogroup 13 (Khan et al., 2011) and whole genome sequencing placed it in the same ancestral population (clade D) than haplogroups 2 (including type II lineage) and 12 (Lorenzi et al., 2016). The same ancestral origin for type II and *Chinese 1* deserves attention. Interestingly, type II strains, which predominate in Europe, are also present in the most western part of Asia (Turkey, Qatar, Iran) and in the Western provinces of China. This suggests a continuum between type II and *Chinese 1* in the Eurasian continent. Data are lacking regarding genotypes circulating in the central part of Eurasia (Eastern European countries, Russia, Uzbekistan, Kazakhstan, Tajikistan, Northern part of India) to observe the transitional and overlapping areas between *Chinese 1* and type II. In these transitional areas, recombination phenomena between these 2 main lineages might be found as observed in North America between haplogroup 12 and type II (Grigg and Sundar, 2009) leading to new genotypes. Another genotype (ToxoDB#20) found in Emirates and Sri Lanka as well as in East Africa (Egypt and Ethiopia) differs from *Chinese 1* by only one marker. Although, it has not been included in the phylogenetic studies by Su et al. (2012) and Lorenzi et al. (2016), it probably belongs to the same ancestral clade together with Type II lineage. The evolutionary history of this ancestral clade gathering type II, *Chinese 1*, and probably ToxoDB#20 could provide an insight on long term migration of *T. gondii*. Three recent studies (Bertranpetit et al., 2016; Lorenzi et al., 2016; Li et al., 2014) that integrate type II and *Chinese I* strains presented phylogenetic analyzes supporting the hypothesis of an earlier divergence of *Chinese 1*. Geostatistical analyses for inferring the putative origin of *T. gondii* point to a South American origin for *T. gondii* and suggest that the parasite has initially spread through the Americas and then colonized Asia and Europe via the Bering Strait, before entering Africa through two different and independent migration routes (Bertranpetit et al., 2016). Concerning Asia, these migration pathways of *Toxoplasma* could partially explain the observed East-West genotype gradient (Figure 2). The migration routes of birds and other intermediate hosts between Asia, Europe and East Africa, which has been suggested to play a role in the propagation of diverse viral and bacterial diseases from Asia to Europe (Jourdain et al., 2007), also support the hypothesis that *Chinese 1* preceded type II. Among intermediate hosts, rodents, as the main prey of felids, play a major role in the *Toxoplasma* cycle. Rodent migrations could have favored the dispersion of genotypes over the large Eurasian continent. Three rodent species, the house mouse (*Mus musculus*), the black rat (*Rattus rattus*), and the brown rat (*Rattus norvegicus*) are the most successful invasive mammals, having colonized most of the global human habitats (Kosoy et al., 2015). All these species originated from Asia and have started their commensalism with the emergence of agrarian civilizations following their worldwide spread through sea-faring and terrestrial trades. The house mouse originated from western Asia (probably Northern India) and diversified in sub-specific lineages, *M. mus musculus*, *M. m. domesticus*, and *M. mus castaneus* with regional diversification in relation to both range expansion in Eurasia and spread by human transport (Cucchi et al., 2005; Suzuki et al., 2013). *M. m. musculus* entered China from Kazakhstan through the north-west border (Xinjiang) and expansion of its population was dated back to 7,150 to 14,300 years ago (Jing et al., 2014). The migration routes of house mice coincide with the colonization routes of modern humans into China, and the expansion times of house mice are consistent with the development of agriculture in southern and northern China (Jing et al., 2014). This

species migrated to North Europe whereas the subspecies *M. m. domesticus* was introduced to South and Western Europe, to Middle East and Arabic Peninsula and North Africa (Bonhomme et al., 2007). *M. m. castaneus* appeared to have migrated through a southern route (Yunnan and Guangxi), approximately 4650–9300 years ago (Jing et al., 2014). The rise of agriculture and trade routes also allowed the global range expansions of black rat from Indian subcontinent and/or Southeast Asia to Western Asia, East Africa and Europe, and lastly to the Americas (Aplin et al., 2011). The black rat was reported from the Levant, 1,500 BC (Audouin-Rouzeau & Vigne, 1994). The brown rat was restricted to northern China and Mongolia and began to disperse southward into Southeast Asia and later worldwide with European sea faring and trades in the last centuries (Puckett et al., 2016).

Human migration and trade exchanges were also proposed to explain a more recent dispersion of *Toxoplasma* genotypes across countries or continents (Lehman et al., 2006; Mercier et al., 2010). Trade exchange between the Eastern and Western part of Eurasia are ancient: the caravans bringing diverse merchandise together with animals along the well-known Silk Road connected Eastern China with Central Asia, the Near East and the Mediterranean countries for as long as 200 BC, during the Han dynasty (Boulnois et al., 2005; Waugh DC) (Fig. 3). The Silk Road has been proposed as a route via which bacterial or parasitic diseases might have spread between Asia and Europe (Morelli et al., 2010; Jiao et al., 2010). A maritime coastal road existed since the first century, linking China to diverse harbors in Southeast Asia, India, Sri Lanka, Iran, Arabic Peninsula, and African horn. New forms of trade exchanges are very active and could perpetuate transmission of *T. gondii* strains between continents. The maritime route could explain the presence of *Chinese 1* in Vietnam (Dubey et al., 2007a), or Sri Lanka (Dubey et al., 2007b). Other examples of strain circulation due to anthropic activities between Old World continents can be encountered: ToxoDB#20 is shared between East Africa (Egypt and Ethiopia) (El Behairy et al., 2013; Dubey et al., 2013), the Emirates and Sri Lanka (Dubey et al., 2007b; Dubey et al., 2010); *Africa 1*, a genotype initially found in Western and Central Africa (Mercier et al., 2010; Ajzenberg et al., 2009) is present in Turkey (Döşkaya et al., 2013), a country known for being a bridge between these continents; the worldwide type III is present both in South Europe, in Iran, in Turkey and Sri Lanka, but nearly absent in China. The island of Sri Lanka is a good example of the diversity of strains that could result from these exchanges: on a relatively small sample of strains (24 strains), 3 type III, 1 *Chinese 1*, and 9 ToxoDB#20 genotypes were described. The few data available from India are not sufficient to appreciate the part of this large subcontinent in this global pattern (Vijaykumar et al., 2016).

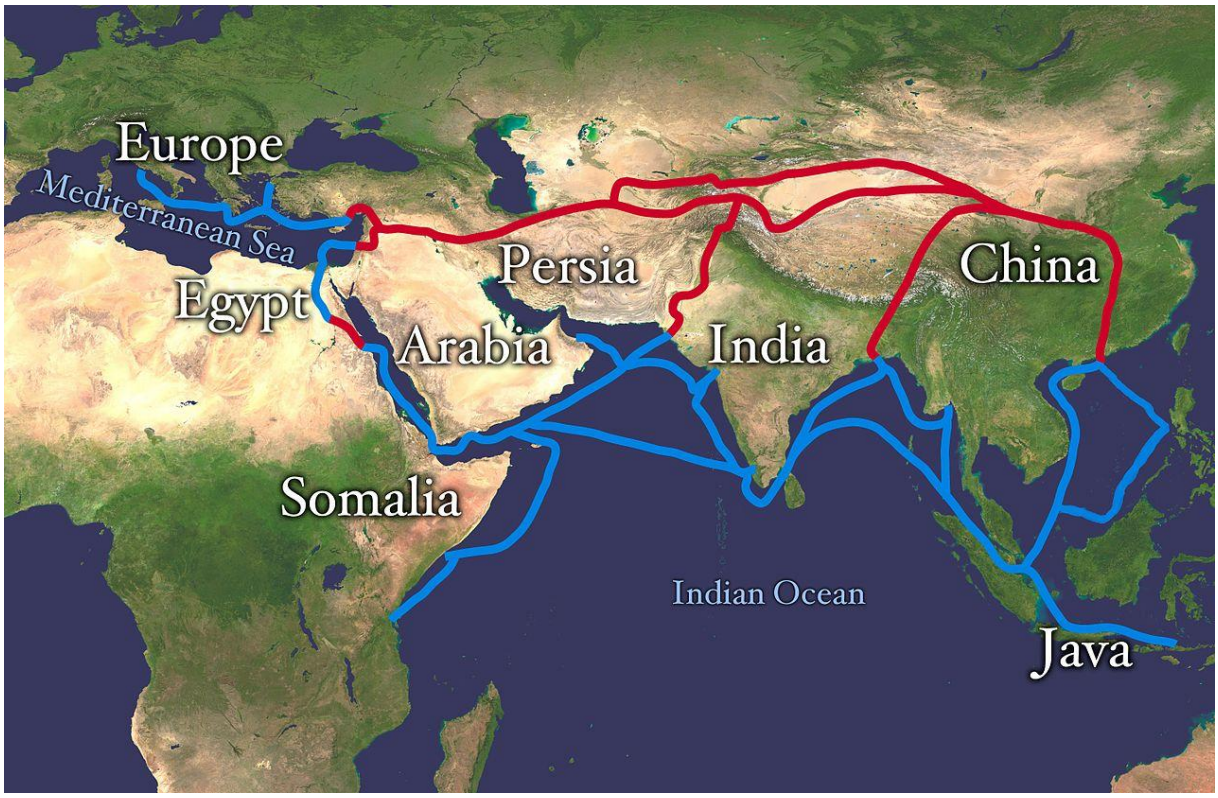

Figure 3: Maritime (blue) and land (red) Silk Roads between Europe and Asia (https://en.wikipedia.org/wiki/Silk_Road).

Type I is the second most common genotype among the 418 samples collected in Asian countries. Although it is considered as a main clonal lineage, it is not frequently isolated worldwide (Ajzenberg, 2010). Outside Asia, it is occasionally found, mainly in South America (Shwab et al., 2014). Even if, generally speaking, some reports of type I genotypes on DNA extracts were suspected to be laboratory contamination due to the large use of RH type I strain in laboratory as positive control for PCR (Ajzenberg, 2010), this should not be the case when mouse-virulent strains were isolated as in Malaysia or Korea (Puvanesuaran et al., 2013a, 2013b; Quan et al., 2008). This relatively large proportion of Type I may suggest an Asian origin for this clonal lineage. Among Asian *Toxoplasma* samples, the majority of Type I samples are found in the most Eastern parts of Asia: from the northeast (South Korea, and the Jilin province of China) to the Eastern provinces of China (Jiangsu, Shandong, Fujian, Shanghai) and to Southeast Asia (Peninsular Malaysia, Myanmar and the neighboring Chinese province of Yunnan). Incomplete genotyping from Japanese and Korean samples confirmed the presence of type I alleles in strains from Far East Asia (Zakimi et al., 2006; Kyan et al., 2012; Kim et al., 2009; Hong et al., 2014). But, Type I strains were also found among samples from the Tibetan plateau (Qinghai, northwestern Chinese province), showing that it may be more widely distributed in Asia. Interestingly, a large part of these type I isolates were encountered in wild animals (33/72) such as bats from Malaysia, Myanmar, Yunnan, Hunan and Jiangxi Chinese provinces or wild boars from the rain forests of Malaysia. From this wild reservoir, it may be transmitted to domestic animals such as free-range ducks in Peninsular Malaysia.

The global genetic diversity is lower than that observed in South America. In South America, 156 different PCR RFLP genotypes (Shwab et al., 2014) were identified among 646 samples whereas in Asia, currently, only 36

different genotypes were encountered in 418 samples. Among the 418 samples, 82.8% (346/418) belong to one of the main clonal lineages (Type I, II, III and *Chinese 1*). However, a large part of the continent remains underexplored. Apart from China, genotype data are available from only 11 Asian countries. A large diversity of host species in the tropical part of Asia may be associated with a higher genetic diversity as observed in other tropical areas (Mercier et al., 2011). Actually, the largest proportion of atypical strains is found in tropical South Asia (Malaysia, Myanmar, Sri Lanka).

This first global picture of *Toxoplasma* genotypes circulating in Asia allowed to make hypotheses about circulation of strains from Asia to Europe, and to East or North Africa through diverse migration pathways (birds, rodents, felids, human activities), but obviously, more studies are needed from unexplored Asian countries to better understand the genetic population structure of *T. gondii* in this continent. Asia exhibits a large variety of ecological conditions that were demonstrated to play a role in *Toxoplasma* population structure, from wild to rural or urbanized areas, tropical or temperate climate, intermediate and definitive host diversity. It should also be remembered that Asia is considered as the birth place of felids (Johnson et al., 2006), the definitive hosts of *T. gondii*. The ancestors of the felid family and of *T.gondii* were both estimated 11-12 million years ago (Johnson et al., 2006; Su et al., 2003). This should open new research perspectives on the possibility of a common or at least partial co-evolution of *T. gondii* and felines that could have had an impact on genetic diversity as described for a number of host-parasite couples (Hume et al., 2003; Hoberg, 2006; Tanabe et al., 2010).

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Chapter IV: Personal works (Materials and methods)

I. Work objectives

There is a lack of information regarding the seroprevalence and genetic diversity in Asia especially in Southeast Asia. In this area, Thailand presents a high diversity of environments. Some studies have been published regarding *T. gondii* seroprevalence in humans and animals but there is no information concerning the genetic diversity of *T. gondii*. This project was focused on the epidemiology of *T. gondii* in Thailand due to its tropical climate, and the diversity of its environments.

The aims of this study were to determine the seroprevalence of free-range chickens in particular biotopes from Thailand and to isolate *T. gondii* strains from the positive free-range chickens. Free-range chickens are considered as a sentinel of *T. gondii* because they fed on the ground and therefore have a high probability to be contaminated with parasite oocysts excreted from cats on the ground.

II. Study areas

To achieve these goals, samplings were performed during 3 field works. The first and second field works were carried out in Kanchanaburi province of Thailand. The third field work was carried out in Lopburi, Nakhonratchasima and Saraburi provinces.

II.1. First and second field works

The project was carried out in 2 villages, Kangpralom and Wangpow of Kanchanaburi province, west region of Thailand, located next to the border with Myanmar. Kangpralom and Wangpow villages present a well preserved rural environment with a high biodiversity. They are crossed by the Kwai river (Figure 18).

Figure 18: Different geographical landscapes of Kanchanaburi province, river Kwai (http://www.routard.com).

II.1.1. Description of Kanchanaburi province

Kanchanaburi is Thailand's third largest province. The most common landscape found in Kanchanaburi is forested mountains. Kanchanaburi is located at latitude 14°00'15" N and longitude 99°32'57" E in the western part of the country (Figure 19). To the west, Kanchanaburi shares a border with Myanmar and has the Tanaowsi Mountain Range as its borderline. It has a total area of 19 483,2 km^2 with a population of nearly $9x10^5$ inhabitants. The majority of populations live in Mueang Kanchanaburi, Thamaka and Thamuang ($4x10^5$ inhabitants). These 3 cities present a mix of urban and rural environments. It is crossed by the Rivers Kwai Yai and Kwai Noi, which emptied into the Bay of Bangkok. There are fertile plains around the meeting point of the Kwai Noi and Kwai Yai Rivers where the town of Kanchanaburi is located. The range of temperatures is between 24.6-38.9°C (https://www.tmd.go.th). The average rainfall is 963 mm/year (Meteorological Department, Ministry of Information and Communication Technology of Thailand).

Figure 19: Thailand map; colored red correspond to Kanchanaburi province. (http://www.visitkanchanaburi.com).

Our study involved 2 small villages (Kangpralom and Wangpow) of Saiyok district in the province of Kanchanaburi (Figure 20). Kangpralom is located in the valley of the Kwai Noi river (Figure 21) at 14°01'28.76" N, 99°13'19.26" E. The landscape is generally rich of forests and mountains. Wangpow village is located at 14°21'44.53" N, 98°53'59.46" E (Figure 22). It is surrounded by forests with a high biodiversity. Wild and preserved environments and ecologies were observed in these villages. Cats and dogs are widely present and freely roam in people's houses and outside, especially in Wangpow.

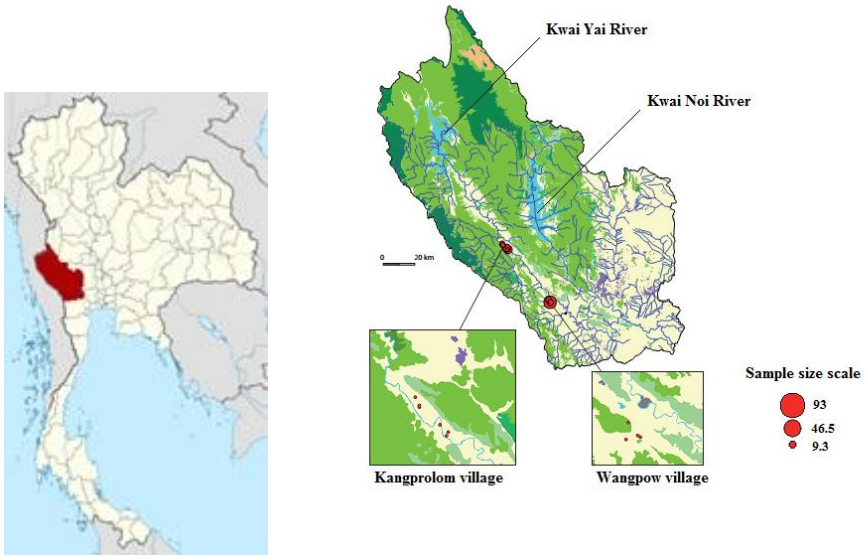

Figure 20: Geographical representation of Kanchanaburi province; Kangpralom and Wangpow villages (realized with the help of Dr. Farid Boumediene, UMR 1094, Limoges University, https://fr.wikipedia.org).

Figure 21: Kangpralom village; A) Inhabitant houses are located near the mountains and the forest; B) Cats are common domestic animals found in almost every house (personal pictures).

Figure 22: Wangpow village; A) Wooden houses nearby forests; B) Several animals can be found in inhabitant houses including consumable and domestic animals such as ducks and dogs (personal pictures).

II.2. Third field work

We decided to collect brains and hearts from free-range chickens bought on markets in 3 provinces in Thailand: Nakhonratchasima, Lopburi and Saraburi. Animal farming is common in these provinces and the main animals found are cattle, pigs and poultry, which are for consumption and for trade. Chickens are raised in the backyard of people's houses where they fed freely on the ground. The majority of the farmers sell their own products on the markets. Fresh products such as vegetables and meat (chicken, ducks …) can be found in the markets. The chicken, duck and quail samples were randomly collected from different markets in these provinces. Occasionally, dead animals (pigeons) found on the roads were collected.

II.2.1. Description of Nakhonratchasima province

Nakhonratchasima is a province situated in Northeast of Thailand. It is situated at latitude 14°58′05″ N and longitude 102°06′00″ E. It covers an area of 20,494 km^2 with $2.6x10^6$ inhabitants. It is located on a plateau about 200-300 meters from sea level. The temperature comprises between 24.4-35.2°C (https://www.tmd.go.th). About 85 % of the populations are farmers (Figure 23). The average rainfall is 1,171 mm/year (Meteorological Department, Ministry of Information and Communication Technology of Thailand). The sampling market is situated at 15°12'21.43" N, 102°02'44.94" E.

Figure 23: A) Nakhonratchasima province (http://www.wikipedia.org); B) Market with a chicken seller (personal picture).

II.2.2. Description of Lopburi province

Lopburi is a province situated in the central part of Thailand. It is about 30 km far from the city of Saraburi (Figure 24). It is located at the latitude 14°47'53" N and the longitude 100°39'14" E. The area of Lopburi is 6,200 km^2 with $7.6x10^5$ inhabitants. The topography of Lopburi is characterized by flat terrain and river valley. It is crossed by Pasak river. The Southwest monsoon has an important influence on the rainfall in Lopburi and the average rainfall is 1,185 mm/year (Meteorological Department, Ministry of Information and Communication Technology of Thailand). The temperature range is between 26.2-36.2°C (https://www.tmd.go.th). The majority of the population is farmer especially animal husbandry and agriculture (Figure 24). The sampling market is found at 14°47'45.45" N, 100°40'18.39" E.

Figure 24: A) Lopburi province (http://www.wikipedia.org); B) Market with a chicken seller (personal picture).

II.2.3. Description of Saraburi province

Saraburi is located in the central part of the country. It is situated at latitude 14°31'42" N and longitude 100°54'35" E. The area of Saraburi is 3,577 km^2 with $6.3x10^5$ inhabitants. The province is crossed by Pasak river. The temperature varied from 24.8-36.2°C depending on the season (https://www.tmd.go.th). The average rainfall is 900mm/year (Meteorological Department, Ministry of Information and Communication Technology of Thailand). The main occupations are farmers and qualified employees (Figure 25). Our sampling market is situated at 14°42'50.06" N, 100°46'54.52" E.

Figure 25: A) Saraburi province (http://www.wikipedia.org); B) Market with a chicken seller (personal picture).

III. Sample collection

III.1. Ethical statement

All experiments with animals (chickens and mice) were carried out in strict and correct conditions in order to ensure the animal welfare according to the Animal Ethics Procedures of Faculty of Tropical Medicine – Animal Care and Use Committee (FTM-ACUC), Mahidol University, Bangkok, Thailand (Permit No. FTM-ACUC 007/2014).

III.2. Blood sampling in chicken

III.2.1. Blood sampling in free-range chickens

Concerning the first field work**,** during 5 months from April to August 2014, 300 blood samples from free range chickens (*Gallus domesticus*) were collected from Kangpralom and Wangpow villages. Based on an estimated seroprevalence of 30% in chickens, we planned to collect 300 free-range chickens in order to increase the possibility in strain isolations. The free-range chickens were firstly identified and recovered from each house and subsequently clustered in an assembly area in order to facilitate the blood-sampling task. Blood samples were drawn from the wing vein (Figure 26). One hundred and fifty-five free-range chickens (7 farms) from Kangpralom and 145 free-range chickens (6 farms) from Wangpow were collected. The ages of chickens were between 2-5 months.

The second field work was conducted also for a 5-month period from May to September 2015. We collected blood samples from 300 free-range chickens from the 2 villages; 6 farms from Kangpralom (138 chickens) and 8 farms from Wangpow (162 chickens) in Kanchanaburi province. The ages of chickens ranged from 3 to 5 months.

III.2.2. Experimentally infected chickens

Positive *Toxoplasma* of chicken serum is needed for the prevalence study. Therefore, 10 free-range household chickens (6-8 weeks old) were obtained and initially examined by IMAT and IFAT to confirm the absence of *T. gondii* antibody before allocating each 5 chickens into group 1 and group 2. Four chickens from both groups were intraperitoneally inoculated with *T. gondii* RH strain tachyzoites: 10,000 toxoplasma and 40,000 toxoplasma, for group 1 and 2, respectively. One chicken from both groups were injected with normal saline as a negative control. All chickens were observed daily and recorded for any clinical sign and symptom of toxoplasmosis. Approximately 1-2 ml of blood samples were collected by puncture of the axillary vein of infected chickens at day 1, 3, 5, 7, 14 and 30 post-inoculation (Figure 26), MAT and IFAT were performed on those collected serum samples to determine whether these experimental chickens develop *T. gondii* IgG antibodies.

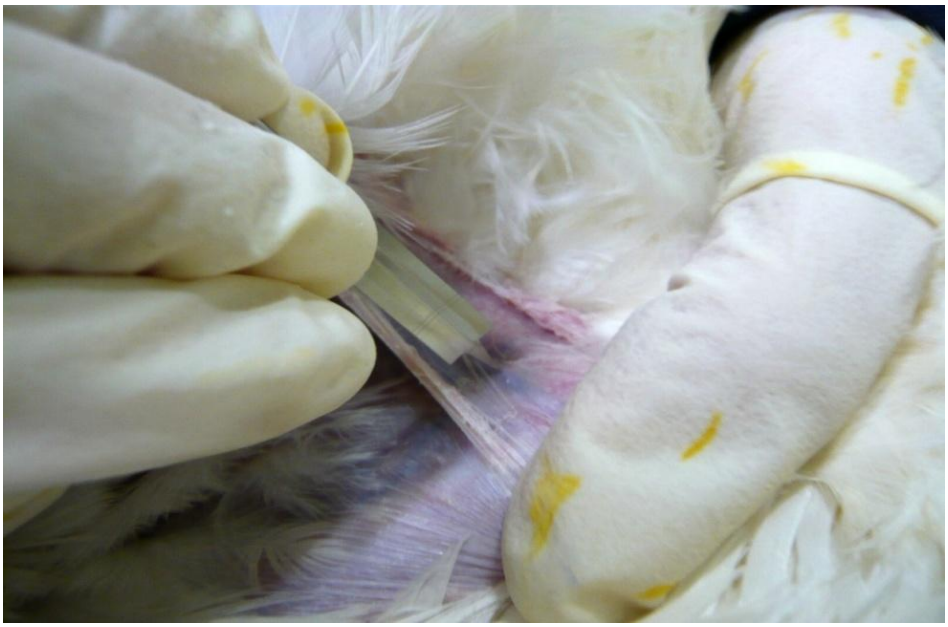

Figure 26: Blood sampling from wing vein of chicken using lancets (personal picture).

Blood samples were centrifuged at 3000 rpm for 10 minutes to separate the serum from the red blood cells (hemoglobin). The serums were recovered in 1.5 ml tubes and kept at -20 °C until use.

III.3. Tissue sampling

III.3.1. First and second field works

Brains and hearts of seropositive chickens were retrieved, put into falcon tubes and ziplock bags without antibiotics, and transported in ice boxes to the laboratory. The transport time was approximately 4 hours from Kanchanaburi to the Laboratory of Mahidol University by car where samples were processed for strain isolation. The experimentation was performed approximately between 8 hours and 5 days after arrival at the laboratory (arrived between 10pm and 11pm at night): 5-8 strain isolations per day (see paragraph VI.1., VI.2.)

III.3.2. Third field work

From the end of April to the end of May 2016, 74 samples (brain and heart) were randomly collected from the markets and from dead animals found on the road of 3 provinces: Lopburi, Nakhonratchasima and Saraburi provinces. Two quails and 10 chickens were collected in Lopburi. Three quails, 3 ducks and 30 chickens were recovered in Nakhonratchasima, and 6 pigeons and 20 chickens in Saraburi. The pigeons were found dead

on the road during the sample collection in Saraburi province. All samples were kept at 4°C in Falcon tubes containing a solution of antibiotics (penicillin/streptomycin 10,000 U and gentamicin 10 mg/ml) in order to prevent bacterial contamination during the transportation. All samples were kept in ice boxes during transportation until storage at Mahidol University laboratory. The transport time was approximately 2-3 hours from Saraburi and Lopburi to the laboratory in Bangkok and 4-5 hours from Nakhonratchasima to the laboratory. The samples were collected 1-2 weeks before sending to Limoges laboratory for isolation. The import authorization for biological samples was obtained from Social Cohesion and Protection of Population Department, Prefecture of Haute-Vienne Limoges (Annex 4). The dirtiest samples were washed in 1 ml of penicillin/streptomycin 10,000 U and gentamicin 10 mg/ml and then soaked in 1 ml of penicillin/streptomycin 10,000 U and gentamicin 10 mg/ml, antibiotics were changed for every 2 days until isolation experiment. The isolation was carried out as below without prior serology (see paragraph VI.1.2., VI.2.).

IV. Serological examinations

All serums were tested for *T. gondii* antibodies by 3 serological tests: IMAT (In house Modified Agglutination Test), CMAT a commercially available Modified Agglutination Test (Toxo-Screen DA®, BioMérieux), and an in-house indirect immunofluorescence antibody test (IFAT).

IV.1. Modified Agglutination Test (MAT): in house MAT (IMAT) and commercial MAT (CMAT)

This serological technique is the most widely used to detect *T. gondii* antibodies in animal serums (Dubey et al., 2015). It is easy to perform.

IV.1.1. In house antigen fabrication for IMAT (laboratory of Parasitology, Limoges University)

Principle of the technique

Tachyzoites from RH strain are grown in the intraperitoneal cavity of mice together with the TG 180 sarcoma cells to obtain the multiplication of tachyzoites in sarcoma cells. This allows to produce between $2x10^8$ and $5x10^8$ tachyzoites per mouse.

Technique

Production of antigen for MAT followed the procedure described by (Desmonts and Remington, 1980). Co-infection RH tachyzoites-TG 180 sarcoma cells was done by mixing approximately 10^7 tachyzoites and 10^7 TG 180 sarcoma cells. This mixture was used to

inoculate intraperitoneally 10 mice (0.2 ml per mouse). About 3 days post inoculation, mice are euthanized and their abdominal fluid is harvested. The abdominal fluid was centrifuged at 700g for 10 minutes. To release the tachyzoites from TG 180 sarcoma cells, a preparation of trypsin solution was necessary. The trypsin solution contained 0.05% of trypsin, phosphate-buffered saline pH 7.2 and 0.2 ml of heparin. The pellet was trypsinized at 37°C in the water bath with agitation. The sarcoma cells were examined under a microscope every 5 minutes until the cells were destroyed to stop the trypsinization. The sediment of parasites was recovered and resuspended in PBS. The centrifugation was done 3 times in PBS at 700g for 10 minutes. The formalin solution was prepared from 6% formaldehyde solution diluted 1:5 in PBS. The formalin solution was added to the pellet and the suspension was kept at 4°C at least 16 hours. After 16 hours of incubation with formalin solution, the formalin solution was removed by 3 successive centrifugations in PBS at 700g for 10 minutes. The suspension of antigen was diluted or concentrated to obtain the final concentration of $3x10^5$ tachyzoites/µl. A conservator, sodium azide (0.1%) was added to the final suspension of antigen and the suspension was stored in vertical position at 4°C for maximum one year. Positive human serum as 400 IU/ml and negative serum were used for the antigen validation.

IV.1.2. MAT procedure

Principle of the technique

The in house and commercial Modified Agglutination Test evaluate the agglutination of a suspension of formalized *T. gondii* tachyzoites (antigen) in the presence of specific IgG antibodies (IgM are destroyed by treatment of serum with 2-mercaptoethanol). In the absence of IgG antibodies, the parasites sediment at the bottom of the well.

Technique

IMAT: The suspension of antigen ($3x10^5$ tachyzoites/µl) was diluted 1:30 in BABS buffer to obtain $1x10^4$ tachyzoites/µl. The 2-mercaptoethanol (14.2 mol/L) was diluted in PBS to obtain a concentration of 0.2 mol/L: 0.35 ml of 2-mercaptoethanol in 24.65 ml of PBS (stock solution). The stock solution was later diluted 1:2 in PBS (working solution) according to the final volume. All serums were diluted in this working solution (1:10, 1:20, 1:100 and 1:800) in the microtiter plates (Table 8A) and 50 µl of diluted suspension of antigen 1:30 were added to each well. The final volume in each well is 100 µl. The microtiter plates were shaken briefly, incubated for at least 7 hours at room temperature, and protected from light and vibration. Titers ≥ 10 were considered as positive. Positive serum as 400 IU/ml and negative

serum obtained by Sabin Feldman Dye Test were used as positive and negative controls respectively.

CMAT: The suspension of antigen used is a commercial antigen (Toxo-Screen DA® kit, Biomérieux SA, France). Stock and working 2-mercaptoethanol solutions were identical to those used for IMAT. Dilution of serum in the working solution of 2-mercaptoethanol was identical (Table 8B). The concentration of the suspension of antigen is ($2.5x10^5$ tachyzoites/µl); the antigen was diluted in 1:5 in colored BABS albumin buffer (red) pH 8.95 to obtain $5x10^4$ tachyzoites/µl. Twenty-five microliters of diluted antigen were added in each well. The final volume in each well is 50 µl. After 5 hours of incubation, sedimentation of tachyzoites was observed. Titers ≥ 10 were considered as positive. Positive and negative controls used were provided in the Toxo-screen DA® kit. This technique was not performed during the fieldwork, but at Limoges laboratory.

Table 8: A) Dilution of serums in 2-mercaptoethanol for IMAT.

	Well 1 (1 :10)	**Well 2 (1 :20)**	**Well 3 (1 :100)**	**Well 4 (1 :800)**
2-ME-PBS	90 µl	50 µl	200 µl	350 µl
Serum	10 µl	50 µl from well 1	50 µl from well 2	50 µl from well 3

50 µl of volume of diluted serum from the previous well

Throw out 150 µl

Throw out 350 µl

Table 8: B) Dilution of serums in 2-mercaptoethanol for CMAT.

	Well 1 (1 :10)	**Well 2 (1 :20)**	**Well 3 (1 :100)**	**Well 4 (1 :800)**
2-ME-PBS	45 µl	25 µl	100 µl	175 µl
Serum	5 µl	25 µl from well 1	25 µl from well 2	25 µl from well 3

25 µl of volume of diluted serum from the previous well

Throw out 75 µl

Throw out 175 µl

Plate reading

A veil of formalized *Toxoplasma* covering more than half of the well was observed for the positive reaction (Figure 27B). Formalized tachyzoites sedimented at the bottom of the well or veil covered less than 50% of the diameter of the well were considered as negative reaction (Figure 27A). The dilution from 1: 10 is considered our positive threshold for both IMAT and CMAT.

Figure 27: Negative and positive reactions of *T. gondii* agglutination by CMAT technique. A) Negative reaction; B) Positive reaction (personal picture).

IV.2. Indirect immunofluorescence Antibody Test (IFAT)

Principle of the technique

This technique was performed according to (Chumpolbanchorn et al., 2009; Udonsom et al., 2010). *Toxoplasma gondii* RH tachyzoites were retrieved from mouse bioassay in saline solution (0.9% NaCl) and the suspension of RH tachyzoites was used as antigen. This preparation was done at Mahidol University. The antibodies present in the serum will bind to the antigens present on *Toxoplasma* tachyzoites deposited on a slide. This antigen-bound antibody is subsequently detected by the use of a secondary antibody, conjugated to a fluorochrome. The deposition of the fluorochrome is observed by a fluorescence microscope.

Technique

Killed tachyzoites were fixed on Teflon printed slides as antigen and kept at -20°C until use. The quantity of *T. gondii* tachyzoites was approximately 5-7 $x10^4$ tachyzoites for each well. The serum is diluted in 2-fold dilutions (1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512 and 1:1024) in diluting buffer (0.01 mol/L phosphate buffer, pH 7.2, 1% BSA, 0.15 mol/L NaCl). Twenty microliters of diluted serum were placed on each well of the slides coated with *T. gondii* tachyzoites. After a 1 hour incubation in humid chamber at 37°C, the slides were flicked to remove the excess of serum, placed in the rinse jar containing 1x FA rinse buffer (0.5 mol/L carbonate buffer, pH 9.0, 0.15 mol/L NaCl) for 3 minutes, and soaked for at least 10 minutes in fresh FA rinse buffer. These slides were drained by pressing the surface around

wells. Ten microliters of the fluorescein conjugated goat anti-chicken IgG (SouthernBiotech USA, optimized concentration at 1:100 from stock solution: 1.0 mg in 1.0 ml PBS/NaN_3) were added on each well. The slides were incubated at 37°C in humid chamber for 1 hour. The slides were later rinsed using FA rinse buffer for 3 minutes and were soaked at least 10 minutes in FA rinse buffer. Finally, the slides were mounted with mounting fluid (pH9.0, glycerol 90.0 ml, DABCO 2.5g, PBS 10.0 ml) and were observed with fluorescence microscope (Axio Vert A1, Inverted Microscope) at x400 magnification. Positive and negative serums were used as positive and negative controls. Positive reaction was defined as a fluorescence of the whole cell membrane of tachyzoites (Figure 28). The dilutions ≥ 16 were considered as positive.

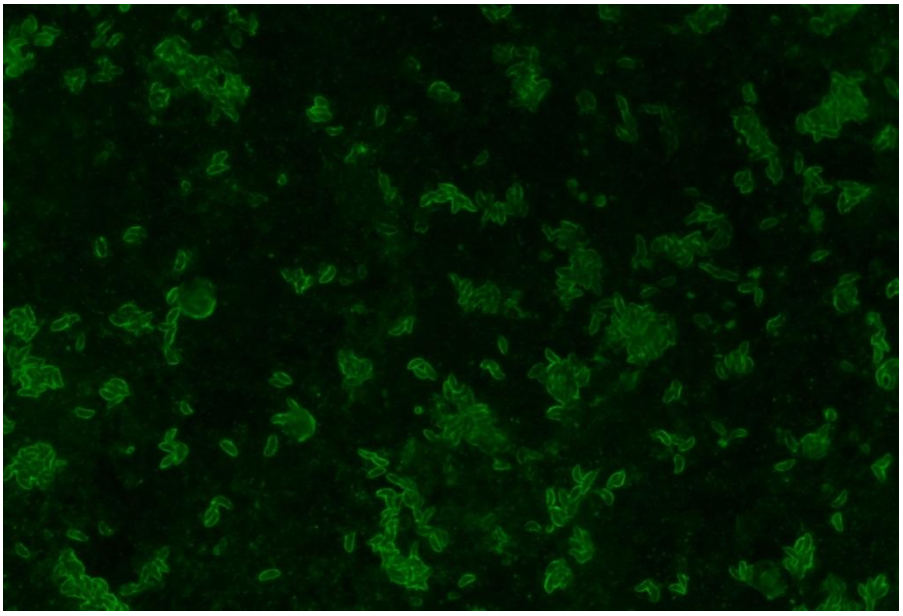

Figure 28: Fluorescent tachyzoites from positive reaction by IFAT (personal picture).

V. Statistical analyses

Statistical analyses were conducted using Fisher's exact test and Chi Square Test ($\chi 2$). An alpha of 0.05 was set for tests of statistical significance. Results were considered significant when $p<0.05$. Statistical analyses were performed using the R x64.3.3.0 software with the two-tailed significance level of 5%. Fisher's exact test was performed to study the correlation between age of chickens and positivity of chickens. Chi Square Test ($\chi 2$) was used to determine the association between study areas and the positivity of chickens. Furthermore, Kappa coefficient was used to estimate the agreement between serological techniques for the detection of *T. gondii* antibodies. The table 9 below showed the accordance and Kappa Coefficient Value.

Table 9: Accordance and Kappa Coefficient Value.

Kappa Value	Accordance
≥ 0.81	Excellent
0.61-0.80	Good
0.41-0.60	Moderate
0.21-0.40	Poor, mediocre
0.00-0.20	Bad
< 0.00	Very bad

VI. *Toxoplasma gondii* isolation methods

For Kanchanaburi samples, due to limited time during fieldwork, we choose to perform *Toxoplasma* isolation by bioassay only on chickens that were seropositive. As some discrepancies were observed between serological methods (see results), strain isolation by bioassay was attempted for chickens that were positive both by IMAT and IFAT (2014 and 2015), chickens positive only by IMAT –whatever the title - (2014 and 2015), and chickens positive only with IFAT whatever the title (2014) or in case of title >1:64 (2015). For market samples (2016), isolation was attempted on all samples.

VI.1. Tissue digestion

Brain and heart of seropositive chicken were pooled and then digested by pepsin digestion (2014) or trypsin digestion (2015 and 2016).

VI.1.1. Pepsin digestion technique (during the first fieldwork in 2014)

The pepsin digestion was performed according to (Dubey, 1998b). The chicken organs (brains, muscles and hearts (approximately 50g)) were cut in small pieces and ground in the blender for 15 seconds (low speed). One hundred and twenty-five ml of NaCl 0.9% were added to the mixture and the mixture was ground with low speed for 15 seconds and with high speed for 15 seconds. The mixture was transferred into a 500 ml glass bottle. The blender was rinsed with 125 ml of 0.9% normal saline and the rinsing liquid was recovered and transferred into the same 500 ml glass bottle already containing the mixture. The pepsin (P7000-100g pepsin from porcine gastric mucosa powder 800-2500 units/mg protein, Sigma-Aldrich Chemie S.a.r.l.) solution containing 1.3g of pepsin, 3.5 ml of HCl and 250 ml of 0.9% normal saline was added to the mixture and then the mixture was incubated 1 hour at 37°C in a waterbath with agitation. After 1 hour of incubation, the mixture was filtered through 2 layers of gauze to remove undigested tissues. The filtrate was transferred into the 250 ml centrifuge tube and was centrifuged 10 minutes at 1200g. The supernatant was removed and the pellet

was recovered in 30 ml of PBS. The mixture was centrifuged again at 1200g for 10 minutes. The pellet was recovered and 20 ml of PBS and 15 ml of 1.2% sodium bicarbonate solution were added to the pellet to neutralize the pH of pellet. The pH was controlled by a pH strip. The centrifugation was done at 1200g for 10 minutes to remove the supernatant. Of the pellet, 200 µl were retrieved for DNA extraction before adding the antibiotics. Finally, 20 µl of Penicillin 10000 Units and Streptomycin 10 mg/ml were added to the pellet (20µl/1 ml of pellet). After 24 hours with antibiotics at 4°C, 0.5 ml of pellet was inoculated intraperitoneally to each mouse; 2 mice were inoculated for each sample.

VI.1.2. Trypsin digestion technique (during the second and third fieldwork, in 2015 and 2016 respectively)

The trypsin digestion was carried out according to (Mercier et al., 2010). Heart and brain of chickens (25g) were cut into small pieces and then ground in the blender for 15 seconds (low speed). One hundred and twenty-five ml of 0.4% trypsin solution containing 0.5g of trypsin (Trypsin 250 from porcine pancreas powder 100g, BD Difco™ BD Biosciences), 125 ml of 0.9% normal saline and 0.5 ml of gentamycin (10mg/ml) were added to the mixture. The mixture was ground for about 2 minutes with the maximum speed (it should not remain identifiable fragments). The mixture was transferred into a sterile glass bottle. The blender was rinsed with the remaining trypsin solution and the rinsing liquid was transferred to the same glass bottle. The mixture was incubated 2-3 hours at 37°C in a waterbath with agitation (be careful not to let 1 hour more the intended digestion time because this can lead to a loss of *Toxoplasma* infectivity). The mixture was filtered through 2 layers of gauze and washed 3 times in normal saline at 1200 g for 10 minutes. The supernatant was discarded after each centrifugation. Of the pellet, 200 µl were recovered for DNA extraction before adding the antibiotics. Twenty microliters of gentamycin 10 mg/ml were added to the last pellet. After 24 hours with antibiotic at 4°C, 0.5 ml of pellet was inoculated intraperitoneally to each mouse (3 mice per sample in 2015 and 2 mice per sample in 2016).

VI.2. Mouse bioassays

All experiments with mice were carried out in strict and correct conditions in order to ensure the animal welfare according to the Animal Ethics Procedures of Faculty of Tropical Medicine – Animal Care and Use Committee (FTM-ACUC), Mahidol University, Bangkok, Thailand (Permit No. FTM-ACUC 007/2014) and according to Animal Ethics Procedures of Limoges University Permit No. (CREEAL 3-7-2012). Female ICR mice *(Mus musculus)* aged 4 weeks (20-25g) were used for bioassays (2014 and 2015) at the Faculty of Tropical

Medicine, Mahidol University. Female Swiss mice *(Mus musculus)* aged 4 weeks (20-25g) were used for bioassays (2016) at Laboratory of Parasitology, Limoges University. Mice were monitored daily post-inoculation for clinical sign observation. Four weeks post inoculation, mouse blood sampling was performed on surviving mice. Blood collection was performed at mouse cheek using the lancet 4 mm (Figure 29). The serological technique (IMAT: 2015 and CMAT: 2016, part IV.1) was performed to check the *T. gondii* infection in mice. Serology was not performed in mice in 2014 due to the lack of antigen for IMAT.

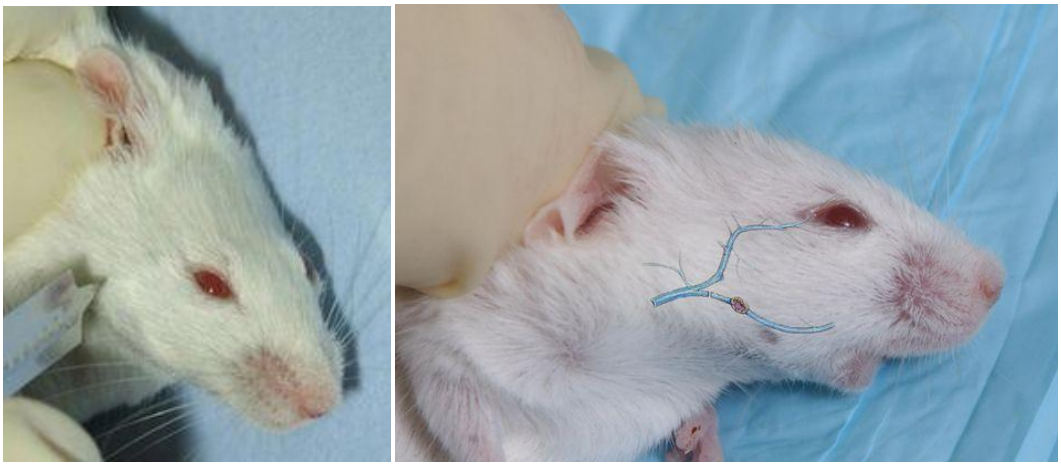

Figure 29: Blood collection from mouse cheek (http://www.bioseb.com/bioseb/francais/Lancette_pour_animaux_GoldenRod.php).

Seropositive mice were euthanized with CO_2 and mouse brains and hearts were retrieved in the 50 ml falcon tubes containing 1.5 ml of saline solution. The brain was ground with a syringe and needle 20G in order to obtain the homogeneous brain suspension. Verification of tissue cysts in mouse brains was performed using microscope. Of the brain and heart homogenous, 200 µl were taken for DNA extraction. The suspensions of mouse brain and heart were further frozen in liquid nitrogen in order to preserve the viability of parasite. The composition of a freezing mix is shown in Table 10. All seronegative mice were euthanized with CO_2 without parasitological control. Frozen mouse brains were further used for the re-inoculation in Limoges (2015).

Table 10: Composition of a freezing mix for 1 sample.

Reagents	Volume (µl)
RPMI	700
10% DMSO*	150**
10% FCS*	150
Suspension of mouse brain	500
Total volume	1500

* % of DMSO and FCS corresponding to the concentration of the reagents in the final freezing volume of 1500 µl; ** DMSO must be the final reagent to be added to the mix to avoid toxicity against cells.

After the distribution of 1500 µl of brain suspension in a cryovial, this one should be placed in the minicryopreservative equipment at room temperature for less than 30 minutes. The mini cryopreservative equipment containing the aliquots was put at -80°C at least 4 hours and then aliquots were rapidly plunged in liquid nitrogen at -196°C.

All experiments should be performed under the microbiological safety hood with sterile conditions.

VII. DNA extraction and DNA detection by quantitative PCR 529 bp

VII.1. DNA extraction

The DNA extraction was done using Minikit Qiagen® (QIAamp DNA Minikit, Qiagen SA, Courtaboeuf, France) according to manufacturer's instructions (Annex 3).

VII.2. DNA detection by quantitative PCR 529 bp

The fragment of the target gene (rep 529), composed of 529 bp, is currently used to detect *T. gondii* DNA in tissues or fluids. The detection by PCR of this gene is very sensitive as it is a multi-copy gene, repeated 200-300 times in the genome of *T. gondii* (Wahab et al., 2010). *T. gondii* primers and specific Taqman probe used for this PCR are listed in Table 11.

Table 11: Primers and probe used for the 529 bp sequence amplification and melting temperatures (TM).

Primers	Oligonucleotide sequences	Nucleotide positions	Genbank N°	Tm
SE	*5-AGGCGAGGGTGAGGATGA*	269-286	AF 146527	57,5 °C
AS	5-TCGTCTCGTCTGGATCGAAT	383-402	AF 146527	59,9 °C
Probe	**Oligonucleotide sequences**	**Nucleotide positions**	**Genbank N°**	**Tm**
TaqMan	5'-(6FAM) CGACGAGAGTCGGA GAGGGAGAAGATGT(BHQla)-3'	308-355	EF 648169	60°C

The probe is labeled at its 5'end by an emitter fluorochrome (FAM Reporter). The 3'end is labeled with a suppressor fluorochrome (Quencher TAMRA). This TAMRA inhibits the FAM reporter emission when they are in proximity to one another. During PCR, if the probe is hybridized to its target, it is hydrolysed by the DNA polymerase (Taq polymerase). The reporter separated from the quencher and emitted a proportional signal to the number of

probes hydrolyzed. It is measurable at the time of elongation. The specificity of the reaction is related to both the primers and the probe that significantly reducing non-specific fluorescence emission due to mismatches. This technique is highly sensitive for *Toxoplasma* infection detection, specific and rapid to perform.

Table 12: Composition of a PCR mix for 1 sample.

Reagents	Volumes (µl)
H_2O	7.1
$MgCl_2$	3.2
Mix Taq	2
Taqman probe	0.2
Primer AS	1
Primer SE	1
UNG (1U/µl)	0.5
DNA	5
Total volume	20

Each DNA sample was tested in duplicate. Each run included a negative control (H_2O) and a positive standard. The machine used for DNA amplification and quantification is the Rotor-Gene 6000: series software, V 1.7 (Corbett Life Science Qiagen Company) with the following amplification cycles:

50 ° C for 2 minutes (Activation phase of the Taq polymerase)

95 ° C for 10 minutes (DNA denaturation phase)

95 ° C for 20 seconds (DNA denaturation)

60 ° C for 30 seconds (Hybridization of the primers and the TaqMan probe) } 50 cycles

VIII. Genotyping by 15 microsatellite markers

Typing with microsatellite markers is used for genetic characterization of the strains. This method of typing is based on a multiplex PCR amplifying in a single assay 15 MS. It classified the strains in types 1, 2, 3 or atypical using the 8 typing markers *TUB2, W35, TgM-A, B18, B17, M33, IV.1* and *XI.1*. and the 7 others markers, *N60, N82, AA, N61, N83, M48* and *M102*, allow an intra-type discrimination (Ajzenberg et al., 2010).

Technique

The mix of 30 primers should be prepared from the primer stock solutions. Twenty-five microliters of each 30 primer stock solutions were taken for the final volume of 750 µl. This mix is kept at -20°C for a year. The 5' end of forward primers of *TUB2, XI.1, B18, N83,*

N61, *M33*, and *M48* were labeled with fluorescein: 6-carboxyfluorescein (6-FAM). At the 5' end of forward primers of *TgM-A*, *B17*, *N82*, *W35*, and *IV.1* were labeled with hexachlorofluorescein (HEX) and the 5'end of forward primers of *AA*, *N60* and *M102* were labeled with 2,7', 8' -benzo-5' -fluoro-2',4,7-trichloro-5-carboxyfluorescein (NED) (Table 13).

Twenty-four microliters of the mix were distributed in the 0.2 ml PCR tubes and DNA extracts were added into the tubes already containing the PCR mix (Table 14).

Table 13: Primers for 15-microsatellite marker technique (adapted from Ajzenberg et al., 2010).

Marker	Primer sequence	Size range (bp)
TUB 2	(F) 5' 6-FAM-GTCCGGGTGTTCCTACAAAA 3' (R) 5' TTGGCCAAAGACGAAGTTGT 3'	287-291
W 35	(F) 5' HEX-GGTTCACTGGATCTTCTCCAA 3' (R) 5' AATGAACGTCGCTTGTTTCC 3'	242-248
TgM-A	(F) 5' HEX-GGCGTCGACATGAGTTTCTC 3' (R) 5' TGGGCATGTAAATGTAGAGATG 3'	203-211
B18	(F) 5' 6-FAM-TGGTCTTCACCCTTTCATCC 3' (R) 5' AGGGATAAGTTTCTTCACAACGA 3'	156-170
B17	(F) 5' HEX-AACAGACACCCGATGCCTAC 3' (R) 5' GGCAACAGGAGGTAGAGGAG 3'	334-366
M33	(F) 5' 6-FAM- TACGCTTCGCATTGTACCAG 3' (R) 5' TCTTTTCTCCCCTTCGCTCT 3'	165-173
IV.1	(F) 5' HEX-GAAGTTCGGCCTGTTCCTC 3' (R) 5' TCTGCCTGGAAAAGGAAAGA 3'	272-282
XI.1	(F) 5' 6-FAM-GCGTGTGACGAGTTCTGAAA 3' (R) 5' AAGTCCCCTGAAAAGCCAAT 3'	354-362
M48	(F) 5' 6-FAM-AACATGTCGCGTAAGATTCG 3' (R) 5' CTCTTCACTGAGCGCCTTTC 3'	209-243
M102	(F) 5' NED-CAGTCCAGGCATACCTCACC 3' (R) 5' CAATCCCAAAATCCCAAACC 3'	164-196
N60	(F) 5' NED-GAATCGTCGAGGTGCTATCC 3' (R) 5' AACGGTTGACCTGTGGCGAGT 3'	132-157
N82	(F) 5' HEX-TGCGTGCTTGTCAGAGTTC 3' (R) 5' GCGTCCTTGACATGCACAT 3'	105-145
AA	(F) 5' NED-GATGTCCGGTCAATTTTGCT 3' (R) 5' GACGGGAAGGACAGAAACAC 3'	251-332
N61	(F) 5' 6-FAM-ATCGGCGGTGGTTGTAGAT 3' (R) 5' CCTGATGTTGATGTAAGGATGC 3'	79-123
N83	(F) 5' 6-FAM-ATGGGTGAACAGCGTAGACA 3' (R) 5' GCAGGACGAAGAGGATGAGA 3'	306-338

Table 14: Composition of a PCR mix for 1 sample.

Reagents	Volumes (µl)
2x QIAGEN Multiplex PCR Master Mix	12.5
Primer mix "Mix 15 MS"	7.5
DNA and RNA free distilled water	4
DNA from mouse brain, tissue digest	3

The DNA amplification takes about 3 hours and 30 minutes. The amplification cycles are indicated in table 15.

Table 15: Amplification cycles of Multiplex PCR 15 MS.

Activation of Taq polymerase		94 °C for 15 minutes
35 cycles	DNA denaturation	94 °C for 30 seconds
	Hybridization of primers	61 °C for 3 minutes
	Elongation	72 °C for 30 seconds
Final elongation		60 °C for 30 minutes
Storage at 4°C		

After the amplification, the mix of 23.5 µl of HiDi™ Formamide and 0.5 µl of GeneScan®-500 [ROX]™ was prepared for a sample. Twenty-four microliters of this mix were placed into a 96 wells plate and then 1 µl of DNA amplified was added into the well containing the mix of HiDi™ Formamide and GeneScan®-500 [ROX]™. The plate was incubated in the water bath at 100°C for 4 minutes and was transferred to a sequencer (AbiPrism 3130 XL, Applied Biosystems). The results of electrophoresis of amplified DNA on an automatic sequencer were analyzed by GeneMapper software V 4.0., Applied Biosystems.

IX. Mouse mortality problem post-inoculation

A high percentage of mortality was observed within 24-48h post inoculation, leading to suspect a bacterial contamination of samples (see Result part). The delay between the sample collection and the isolation was long enough to allow the development of bacteria in our samples. Bacterial contamination was evoked as being the main cause of mouse mortality. Isolation of bacteria was performed in order to reveal the presence of bacteria in our samples and to better understand the real cause of mouse mortality.

IX.1. Analysis of bacterial contamination of samples

A bacterial analysis of samples was performed on randomly selected digestion pellets of samples collected from Lopburi, Nakhonratchasima and Saraburi provinces for which the inoculated mice were dead: 3 pellets from chickens in Saraburi, 2 pellets from chickens in Lopburi and 1 pellet from duck in Nakhonratchasima.

The digestion pellets were spread out on Petri dishes containing nutrient agar (non-selective medium). All species of bacteria could grow in this growth medium. The bacteria, which grew on the non-selective medium, were further inoculated on a selective medium (blood agar). This selective medium can isolate hemolytic bacteria such as *Streptococcus, Pneumococcus, Staphylococcus, Listeria and Erysipelothrix.*

The isolated bacteria were identified by Dr. Olivier Barraud and Dr. Thomas Jové of UMR INSERM 1092 (Anti-Infectieux: supports moléculaires des résistances et innovations thérapeutiques) using mass spectrometry (Vitek MS – BioMérieux®).

X. Organization charts of 3 field works

Figures 30 and 31 represent the important steps allowing the realization of successive 3 field works.

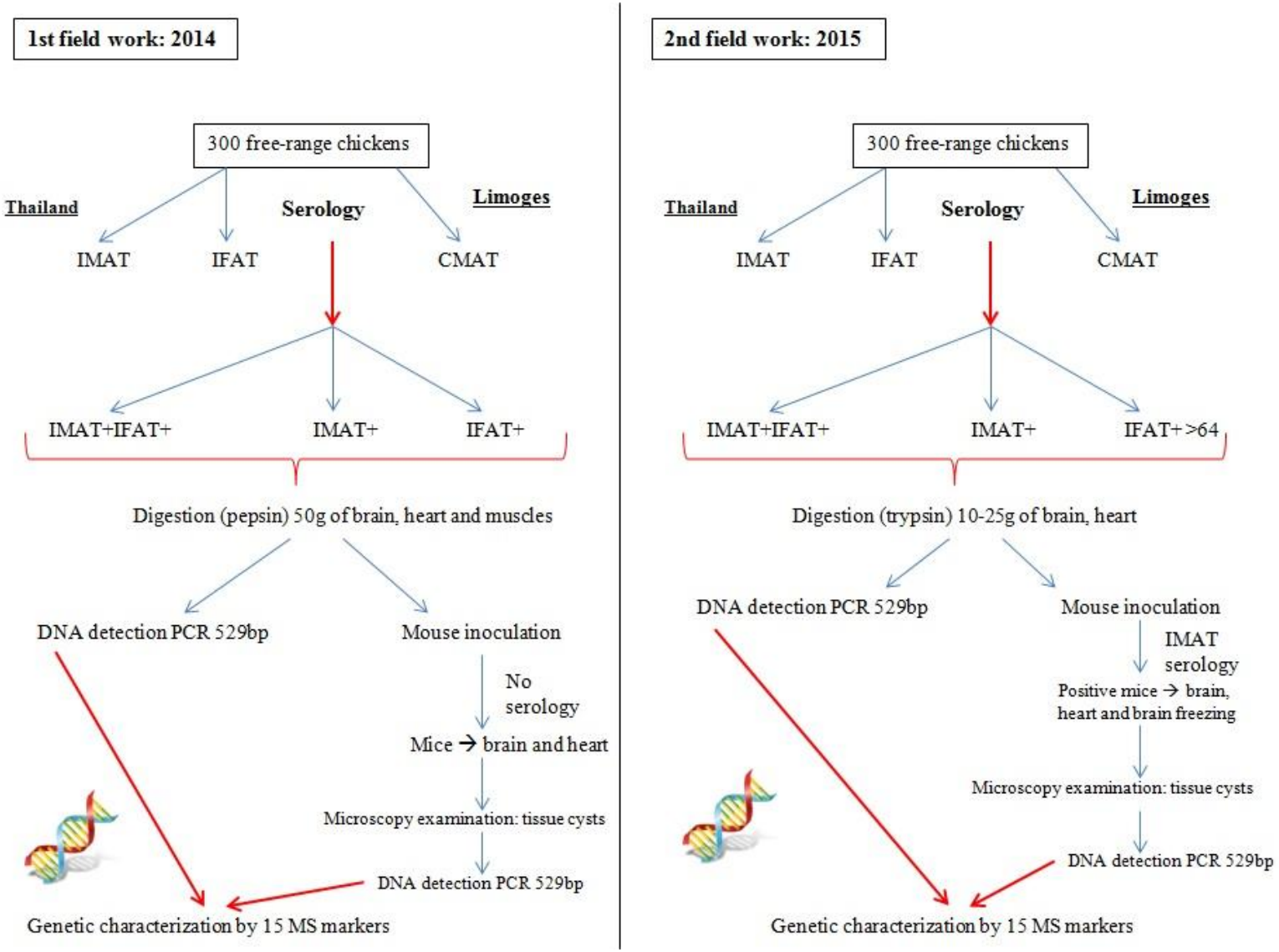

Figure 30: Organization charts of first and second field works

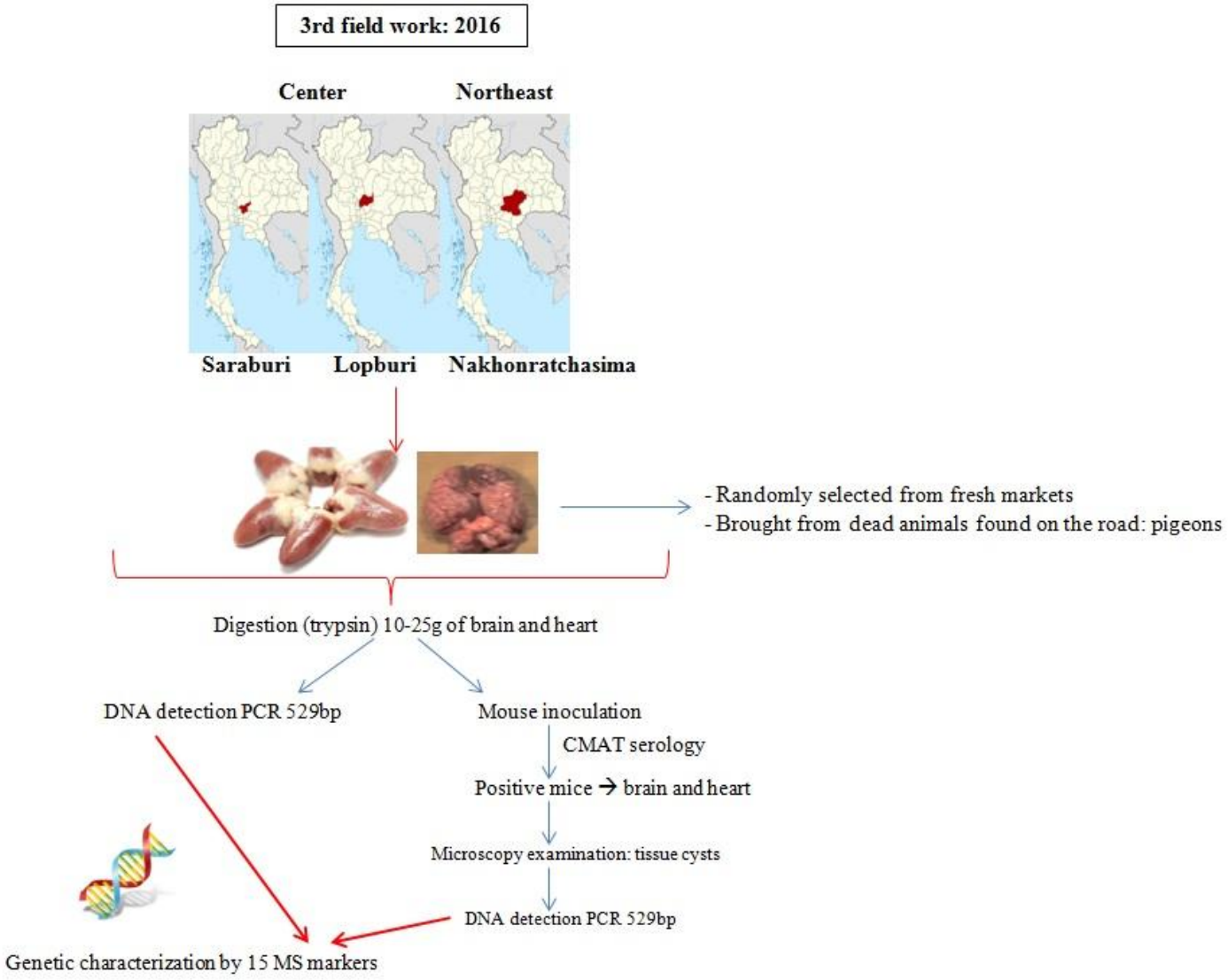

Figure 31: Organization chart of third field work

Chapter V: Results

I. Sero-epidemiology

I.1. Seroprevalence in free-range chickens from Kangpralom and Wangpow villages in Kanchanaburi province (2014 and 2015)

The overall seroprevalence for the 2 years from 600 free-range chickens (2014 and 2015) was 11.5% (95% CI: 8.9-14.1), 17.7% (95% CI: 14.6-20.7) and 33.0% (95% CI: 29.2-36.8), by IMAT, CMAT and IFAT respectively. Our samples were collected from 2 villages, which presented slightly different environments. There was no association between study areas and the positivity of chickens either by IMAT or by IFAT ($p>0.05$). Only CMAT test showed an association between the study areas and the positivity of chickens with slightly significant p value ($p=0.04$) (Table 16).

Table 16: Seroprevalence of free-range chickens from Kangpralom and Wangpow villages in Kanchanaburi province (2014 and 2015).

Chicken N°	Farm N°	N° of collected chickens	N° of positive chickens (IMAT≥1:10)	N° of positive chickens (CMAT≥1:10)	N° of positive chickens (IFAT≥1:16)
Kangpralom					
2014-K1-5	1	5	0	0	0
2014-K6-55	2	50	4	5	14
2014-K56-97	3	42	4	7	10
2014-K98-103	4	6	0	0	1
2014-K104-120	5	17	2	2	1
2014-K121-135	6	15	0	0	3
2014-K136-155	7	20	3	3	1
2015-K1-30	1	30	1	8	24
2015-K31-68	2	38	6	11	20
2015-K69-97	3	29	2	0	12
2015-K98	4	1	0	0	0
2015-K99-116	5	18	1	4	3
2015-K117-138	6	22	4	2	2
Total	13	293	27	42	91
% (95% CI)			9.2% (5.9-12.5)	14.3% (10.3-18.3)	31.1% (25.8-36.4)
Wangpow					
2014-W1-38	1	38	1	4	5
2014-W39	2	1	1	1	0
2014-W40-73 and W76-96	3	55	1	3	8
2014-W74-75	4	2	2	2	0
2014-W97-120	5	24	4	4	11
2014-W121-145	6	25	2	3	17
2015-W1-20	1	20	3	4	8
2015-W21-40	2	20	5	6	15
2015-W41-70	3	30	11	9	14
2015-W71-79	4	9	0	1	4
2015-W80-99	5	20	0	5	11
2015-W100-140	6	41	11	18	11
2015-W141-145	7	5	0	1	2
2015-W146-162	8	17	1	3	1
Total	14	307	42	64	107
% (95% CI)			13.7% (9.9-17.5)	20.8% (16.3-25.3)	34.9%(29.6-40.2)
p-value between villages			**0.09**	**0.04**	**0.34**
Total number	**27**	**600***	**69**	**106**	**198**
% (95% CI)			**11.5% (8.9-14.1)**	**17.7% (14.6-20.7)**	**33.0%(29.2-36.8)**

* Two serum samples were lacking for CMAT

I.2. Experimentally infected chickens

Following *T. gondii* inoculation, chickens did not show any clinical sign and symptom that specific or suggestive to toxoplasmosis, however, one chicken in group 2 (higher dose of inoculation) died before day 30. The earliest detection of *T. gondii* antibodies was at 7 days post-inoculation in one chicken (C4) in group 1 with titer of 1:32 and another (C9) in group 2 with titer of 1:256 by IFAT. Although, IMAT could not detect any positive chicken in group 1 within 7 days post inoculation, one (C9) in group 2 showed seropositive titer at 1:100 (Table 17). The antibody was shown in all infected chickens by day 14 showing titers ranging from 1:64 to 1:1024 by IFAT and from 1:10 to 1:400 by IMAT. At day 30, more than half of seropositive chickens showed stable or lower titer by both IFAT and IMAT. All control chicken sera were found negative by both serological methods.

Table 17: *T. gondii* antibody titers detected by IFAT and IMAT in experimental chickens (n = 10).

Experimental chicken (code)	*T. gondii* inoculation (cells/ml)	Period of serum sample collected after inoculation (day)											
		IFAT titer (cut off 1:16)						IMAT titer (cut off 1:10)					
		1	3	5	7	14	30	1	3	5	7	14	30
Group 1													
C1	10,000	-	-	-	-	1024	512	-	-	-	-	20	20
C2	10,000	-	-	-	-	512	512	-	-	-	-	40	20
C3	10,000	-	-	-	-	1024	512	-	-	-	-	20	10
C4	10,000	-	-	-	32	128	128	-	-	-	-	10	20
C5	NSS[1*]	-	-	-	-	-	-	-	-	-	-	-	-
Group 2													
C6	40,000	-	-	-	-	1024	1024	-	-	-	-	400	20
C7	40,000	-	-	-	-	512	128	-	-	-	-	400	80
C8	40,000	-	-	-	-	64	64	-	-	-	-	400	20
C9	40,000	-	-	-	64	256	death	-	-	-	100	400	death
C10	NSS[1*]	-	-	-	-	-	-	-	-	-	-	-	-

* Chickens inoculated with normal saline as negative control

I.3. Statistical analyses

I.3.1. Comparison of age/positivity of chickens (2014 and 2015)

The free-range chickens were grouped into 2 age classes as showed in (Table 18, 19 and 20). If we consider IMAT, CMAT and IFAT results, chicken ages did not seem to have an influence on the *T. gondii* infection in chickens (p>0.05).

Table 18: IMAT results in free-range chickens classified by age classes.

Age classes [months]	Neg (%)	Pos (%)	Total	p-value
[2 ; 3]	454 (88.0)	62 (12.0)	516	0.45
[4 ; 5]	77 (91.7)	7 (8.3)	84	
Total	531 (88.5)	69 (11.5)	600	

Table 19: CMAT results in free-range chickens classified by age classes.

Age classes [months]	Neg (%)	Pos (%)	Total	p-value
[2 ; 3]	424 (82.5)	90 (17.5)	514	0.75
[4 ; 5]	68 (81.0)	16 (19.0)	84	
Total	492 (82.3)	106 (17.7)	598*	

*Two serum samples were lacking for CMAT

Table 20: IFAT results in free-range chickens classified by age classes.

Age classes [months]	Neg (%)	Pos (%)	Total	p-value
[2 ; 3]	349 (67.6)	167 (32.4)	516	0.24
[4 ; 5]	53 (63.1)	31 (36.9)	84	
Total	402 (67.0)	198 (33.0)	600	

I.3.2. Concordance between serological techniques

If we consider the 3 serological techniques used, there was a moderate concordance between IMAT and CMAT whereas there was no substantial concordance between

IFAT/IMAT and IFAT/CMAT. Kappa allows defining the concordance between 2 serological tests considering as true positive and true negative those presenting the same qualitative results by the 2 techniques.

- The Kappa coefficient was 0.47, indicating a moderate concordance between IMAT and CMAT (Table 21).

Table 21: Number of positive and negative chickens and Kappa Coefficient calculated for the concordance between IMAT and CMAT.

IMAT/CMAT	Neg	Pos	Total	Kappa coefficient
Neg	472	58	530	0.47
Pos	20	48	68	
Total	492	106	598	

Concordance – IMAT - CMAT (Figure 32):
Sensitivity = 0.45 (45.0%)
Specificity = 0.95 (95.0%)

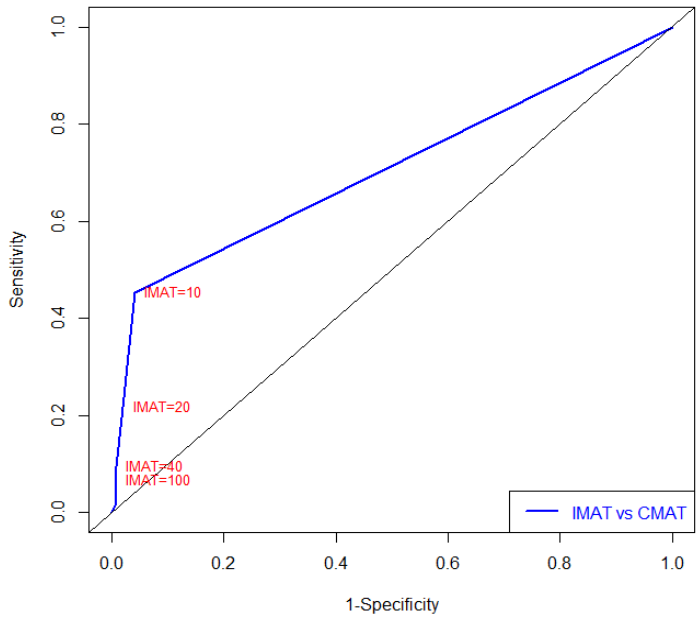

Figure 32: Receiver operating characteristics (ROC) analysis of IMAT vs. CMAT. Black line is a diagonal line from bottom left to right hand corner showing the plot of the test; Blue line corresponding to the sensitivity and specificity between IMAT vs CMAT tests.

- The Kappa coefficient was 0.09, indicating that there was no concordance between IFAT and CMAT tests (Table 22).

Table 22: Number of positive and negative chickens and Kappa Coefficient calculated for the concordance between IFAT and CMAT.

IFAT/CMAT	Neg	Pos	Total	Kappa coefficient
Neg	342	60	402	0.09
Pos	150	46	196	
Total	492	106	598	

Concordance – IFAT - CMAT (Figure 33):
Sensitivity = 0.43 (43.0%)
Specificity = 0.69 (69.0%)

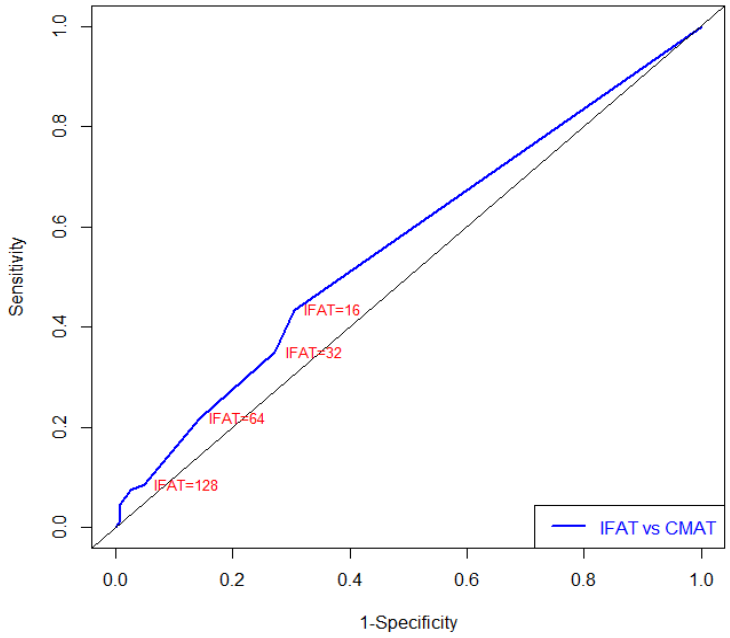

Figure 33: Receiver operating characteristics (ROC) analysis of IFAT vs. CMAT. Black line is a diagonal line from bottom left to right hand corner showing the plot of the test; Blue line corresponding to the sensitivity and specificity between IFAT vs CMAT tests.

- The Kappa coefficient was 0.02, meaning that there was no concordance between IMAT and IFAT tests (Table 23).

Table 23: Number of positive and negative chickens and Kappa Coefficient calculated for the concordance between IFAT and IMAT.

IFAT/IMAT	Neg	Pos	Total	Kappa coefficient
Neg	359	43	402	0.02
Pos	172	26	198	
Total	531	69	600	

Concordance – IFAT - IMAT (Figure 34):

Sensitivity = 0.36 (36.0%)

Specificity = 0.67 (67.0%)

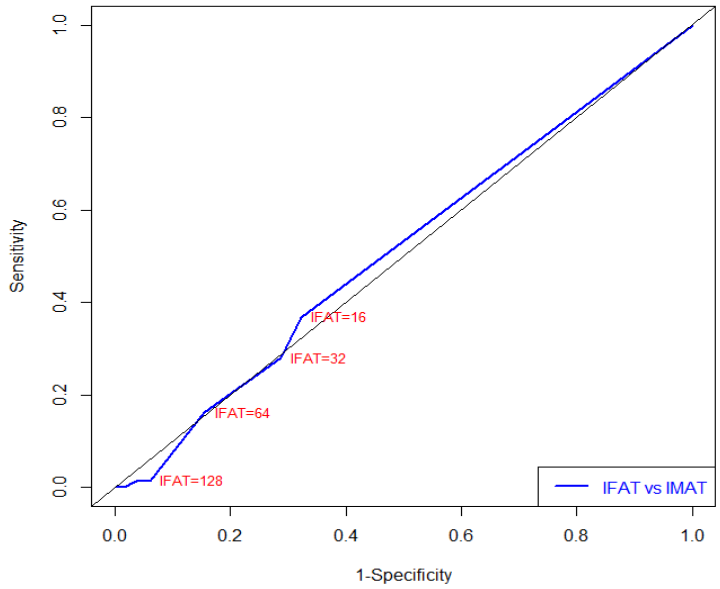

Figure 34: Receiver operating characteristics (ROC) analysis of IFAT vs. IMAT. Black line is a diagonal line from bottom left to right hand corner showing the plot of the test; Blue line corresponding to the sensitivity and specificity between IFAT vs IMAT tests.

II. Isolation attempts

The results of the 3 field works are presented successively: 2014, 2015 and 2016.

II.1. First field work in 2014

Out of 85 seropositive chickens by IMAT and/or IFAT, the isolation of *T. gondii* was attempted only on 65 chickens as 20 seropositive chickens died or disappeared before returning to the study areas. The reported cause of death was fight between chickens. These seropositive chickens corresponded to:

1. 9/10 chickens positive both by IMAT and IFAT
2. 13/14 chickens positive only by IMAT (for all titers)
3. 43/61 chickens positive only by IFAT (for all titers)

II.1.1. Direct detection of Toxoplasma DNA in pepsin digestion pellets from brains, hearts and muscles of seropositive chickens (2014)

A DNA extraction was performed on the 65 digestion pellets obtained after pepsin digestion (II.1), 27 from Wangpow village and 38 from Kangpralom village. *T. gondii* DNA detection with a real time PCR targeting rep529 was positive in only 5 samples (Table 24). DNA detection was performed in duplicate. Genotyping using the 15 microsatellite markers was attempted on all positive DNA but no genotype was identified due to low amount of toxoplasmic DNA in these samples (Table 31).

Table 24: Samples with positive *Toxoplasma* DNA detection in digestion pellets (2014).

Chicken number	Positive serology test	PCR 529 bp (Ct)
W74	IMAT 1:10	39.2/-
W75	IMAT 1:10	39.5/-
W133	IFAT 1:16	38.9/39.7
W143	IFAT 1:32	38.4/-
K59	IFAT 1:64	40.9/-

II.1.2. Bioassay in mice (2014)

According to the feasibility and to time constraint, bioassays were performed only on 16 seropositive chickens (12 from Wangpow village and 4 from Kangpralom village): 7 IMAT/IFAT seropositive chickens (W27, W114, W144, W145, K26, K30 and K34), 6 seropositive chickens by IMAT only (W39, W74, W75, W97, W110 and K20) and 3 seropositive chickens by IFAT only (W115, W116 and W117). From these 16 digestion

pellets, 2 were positive in direct DNA detection (W74 and W75). Sixteen digestion pellets were inoculated in mice (2 mice/digestion pellet). Of the 32 mice inoculated, 14 died 24h after inoculation. Brains and hearts were taken from the 18 remaining mice 4 weeks post-inoculation corresponding to 11 free-range chickens: 2 free-range chickens from Kangpralom village and 9 free-range chickens from Wangpow village. The serological test was not performed on the remaining mice due to the lack of *Toxoplasma* antigen. Microscopic examination of brains was negative. The quantitative real time PCR 529 bp, done in duplicate, revealed only 6 positive DNA from mouse brains and mouse hearts corresponding to 4 different free-range chickens (Table 25). These 4 positive chickens after mouse inoculation were different from the 5 positive chickens detected by direct DNA detection on digestion pellets. A genetic characterization was attempted for all these 6 positive DNA and no strain was identified (Table 31). Actually, one positive DNA of mouse heart (W114 M1) were characterized with all 15 MS markers (*TUB2, W35, TgM-A, B18, B17, M33, IV.1, XI.1, N60, N82, AA, N61, N83, M48* and *M102*), but comparison with the laboratory RH strain type 1 revealed a probable contamination.

Table 25: Positive *Toxoplasma* DNA detection in brains and hearts of mice inoculated with digestion pellets of chicken (2014).

Samples	**Positive serological test (chicken)**	**Mouse 1 brain PCR 529 bp (Ct)**	**Mouse 1 heart PCR 529 bp (Ct)**	**Mouse 2 brain PCR 529 bp (Ct)**	**Mouse 2 heart PCR 529 bp (Ct)**
W97	IMAT 1:40	NA	NA	41.5/-	-/-
W110	IMAT 1:10	-/-	42.3/-	-/-	-/-
W114	IMAT 1:10/ IFAT 1:32	42.2/43.2	29.0/28.9*	-/-	40.3/-
W117	IFAT 1:128	-/-	-/-	40.5/-	-/-

*RH contamination

This field work study did not allow achieving our goals of live parasite isolation and genetic characterization due to low concentration of DNA extracted. After analyzing these first negative results, we needed to carry out another field work in order to obtain better results.

II.2. Second field work in 2015

Based on 156 seropositive free-range chickens by MAT and/or IFAT, *T. gondii* isolation was performed on 72 seropositive chickens as mentioned below:

1. 16/16 chickens positive both by IMAT and IFAT
2. 29/29 chickens positive by IMAT (for all titers)
3. 27/111 chickens positive by IFAT (titers > than 1:64) (instead of all titers in 2014)

The other main differences between the two field works were: the use of trypsin digestion instead of pepsin digestion, digestion performed only on brain and hearts of chickens (and not on muscle), bio-assays attempted on all the selected chickens, inoculation of 3 mice per chicken instead of 2, control of the serology of inoculated mice.

II.2.1. Direct detection of Toxoplasma DNA in trypsin digestion pellets from brains and hearts of seropositive chickens

A quantitative real time PCR targeting rep529 to detect *Toxoplasma* DNA was performed on these 72 pellets obtained after trypsin digestion of heart and brains of seropositive chickens: 26 from Kangpralom village and 46 from Wangpow village. *Toxoplasma* DNA detection was positive for 23 digestion pellets, with Ct values between 20 to 37, indicating for some of them a high amount of toxoplasmic DNA in the samples. Unfortunately, the genetic characterization by 15 microsatellite markers showed that all positive samples were contaminated by the laboratory RH strain type 1.

II.2.2. Bio-assays in mice (2015)

Mouse bioassays using trypsin digestion pellets were performed for all 72 seropositive chickens (II.2). To increase the chance of strain isolation, 3 mice were inoculated for each seropositive chicken instead of 2 mice per seropositive chicken in 2014. Four weeks post-inoculation, IMAT serology was carried out for all 216 inoculated mice (Table 26). In Kangpralom village, 32 mice corresponding to 21 seropositive chickens were seropositive for *T. gondii*. In Wangpow village, 39 mice corresponding to 24 seropositive chickens were positive for *T. gondii* infection. IMAT results are shown in (Table 26).

Table 26: Detection of antibodies in mice inoculated with trypsin digestion pellets of 72 seropositive chickens (IMAT results) in 2015.

Samples Kangpralom	Mouse 1	Mouse 2	Mouse 3	Samples Wangpow	Mouse 1	Mouse 2	Mouse 3
K5	1:800	1:800	1:800	W9	-	-	-
K6	1:100	1:800	1:800	W15	-	-	1:10
K7	1:100	1:100	1:800	W17	1:100	1:100	-
K23	-	1:800	-	W18	-	-	-
K25	-	-	1:10	W20	-	-	-
K27	-	1:800	-	W21	-	-	-
K28	-	1:800	1:800	W23	1:100	-	1:100
K31	-	-	1:800	W26	-	-	-
K32	-	1:800	-	W28	-	1:10	1:10
K33	-	-	-	W31	1:100	1:100	1:100
K50	-	1:20	-	W34	-	-	-
K61	-	1:10	-	W35	-	-	-
K62	-	-	-	W36	-	-	1:800
K65	-	-	1:10	W40	-	-	-
K66	-	-	1:10	W42	-	-	-
K68	-	1:20	-	W45	-	-	1:100
K76	1:800	-	1:800	W49	-	-	-
K85	-	1:800	1:800	W50	-	-	1:10
K93	-	-	1:800	W52	-	-	-
K94	-	-	-	W54	1:100	1:100	-
K95	-	1:800	-	W55	-	-	-
K113	-	-	-	W56	-	-	-
K121	-	-	1:800	W57	-	-	-
K129	-	1:100	1:800	W61	-	-	1:100
K137	-	-	-	W67	-	1:100	1:100
K138	-	1:800	1:800	W69	-	-	-
				W71	1:100	1:20	1:100
				W74	-	-	-
				W91	-	-	1:800
				W93	-	-	-
				W95	1:800	1:800	-
				W105	-	-	-
				W110	-	-	1:800
				W111	-	-	-
				W118	-	1:100	-
				W121	-	-	1:800
				W126	-	1:100	1:100
				W127	-	-	-
				W129	-	-	1:800
				W130	1:800	1:800	-
				W132	1:800	1:800	1:800
				W136	-	1:800	1:800
				W139	-	-	1:800
				W140	-	-	-
				W143	-	-	-
				W149	-	1:800	-

Brains and hearts of these 71 seropositive mice corresponding to 45 seropositive chickens were retrieved to verify the presence of live parasite by microscopic examination and no *T. gondii* cysts were observed. *Toxoplasma* DNA was detected in 10/71 brains of seropositive mice (Table 27). Although the ct values were very high indicating a very low amount of DNA, a genetic characterization was attempted and revealed no genotype of *T. gondii*. A sub inoculation into new mice in Limoges was carried out from all these 71 mouse brains (mouse brains, which were frozen in Mahidol with DMSO): 3 mice per sample. The IMAT serology was performed 4 weeks post-inoculation and this sub inoculation was negative.

Table 27: Positive *Toxoplasma* DNA detection in brains of mouse brains inoculated with trypsin digestion pellets of 45 chickens (2015).

Samples	Positive serological test (chicken)	Mouse 1 PCR 529 bp (Ct)	Mouse 2 PCR 529 bp (Ct)	Mouse 3 PCR 529 bp (Ct)
K5	IFAT 1:512	-/-	38.4/-	-/-
K7	IFAT 1:256	-/-	38.3/-	-/-
K23	IFAT 1:128	NA	37.9/40.4	NA
K76	IFAT 1:64	-/-	NA	38.8/-
K95	IMAT 1:10	NA	39.3/-	NA
K121	IMAT 1:20	NA	NA	38.1/39.5
K129	IMAT 1:20	NA	-/-	45.0/-
W118	IMAT 1:20	NA	38.3/-	NA
W126	IMAT 1:10	NA	-/-	35.7/36.8
W132	IMAT 1:10	45.9/-	-/-	-/-

Only 3/71 DNA extracted from mouse hearts were found positive for *T. gondii* (Table 28). The brains of 2 of them (K129 and W126) were also DNA positive. The genetic characterization was carried out for these 3 positive DNA but no genotype of *T. gondii* was identified due to low quantity of *Toxoplasma* DNA.

Table 28: Positive *Toxoplasma* DNA detection in hearts of mice inoculated with trypsin digestion pellets of 45 chickens (2015).

Samples	Positive serological test (chicken)	Mouse 1 PCR 529 bp (Ct)	Mouse 2 PCR 529 bp (Ct)	Mouse 3 PCR 529 bp (Ct)
K6	IFAT 1:256	NA	NA	48.8/-
K129	IMAT 1:20	NA	41.8/45.4	-/-
W126	IMAT 1:10	NA	-/-	33.8/34.2

This second field work study did not achieve our aim of identifying the genotype of *T. gondii*. There was no substantial agreement between serological test and DNA detection as shown from our results (Table 26, 27 and 28). Positive chickens by serological tests were not positive for DNA detection. Same result was observed in bioassay. Seventy-one mice were

seropositive to *T. gondii* whereas only 10/71 among them had positive DNA from brains and 3/71 had positive DNA from hearts. Another supplementary work was needed to complete our results.

II.3. Third field work in 2016

Design of this study was different from the first and second field works. Samples of free-range chickens (brain and heart) were randomly selected on diverse Thai markets without previously testing the positivity of samples by serological test. All the 74 samples were directly brought from Thailand to Limoges, France by plane and train (approximately 24 hours to arrive at Laboratory of Parasitology, University of Limoges) with a maximum of 10 days of additional delays for the last sample processing.

II.3.1. Direct detection of Toxoplasma DNA in trypsin digestion pellets from brains and hearts

From 74 samples, 8 positive DNA were obtained: 3/26 in Saraburi province (2 from pigeons and 1 from chicken), 1/12 from chicken in Lopburi province, 4/36 in Nakhonratchasima province (1 from quail and 3 from chickens). The DNA detection was performed in duplicate for each sample (Table 29). The genetic characterization was carried out for all positive DNAs but no genotype was identified.

Table 29: Positive *Toxoplasma* DNA detection in digestion pellets (2016).

Samples	Locations	PCR 529 bp (Ct)
P1 pigeon	Saraburi	41.3/-
P3 pigeon	Saraburi	39.2/-
Q10 quail	Nakhonratchasima	44.7/-
C20 chicken	Saraburi	49.9/-
C28 chicken	Lopburi	34.9/-
C35 chicken	Nakhonratchasima	46.9/-
C42 chicken	Nakhonratchasima	34.3/-
C51 chicken	Nakhonratchasima	39.9/-

II.3.2. Bio-assays in mice (2016)

These 74 digestion pellets were inoculated in mice (2 mice/sample). One hundred and nineteen mice (80.4%) died 24-48 hours after inoculation. It remained 29 mice related to 17 samples, which survived 3-4 weeks after inoculation: 11 mice corresponding to 6 pigeon samples, 10 mice corresponding to 5 quail samples and the 8 remaining mice corresponding to 6 chicken samples. A serological test using the commercial CMAT was performed on these 29 surviving mice and revealed only 1 seropositive mouse with low titer (1:20). This

seropositive mouse has been inoculated with C7 chicken sample from Saraburi province. Brain and heart were recovered from this seropositive mouse inoculated from digestion pellet (C7 chicken). Microscopic control of brain for tissue cyst detection was negative. DNA was extracted from these 2 organs. Mouse brain DNA showed positive result with Ct 35.4/37.1 and mouse heart DNA gave Ct 32.9/32.7. The genetic characterization was performed on these 2 DNA using 15 microsatellite markers, but no strain was identified, due to the low amount of parasite in the sample, only 1 typing marker (*B18*) exhibited allele I or III for C7 mouse heart. Fresh C7 mouse brain and heart were then sub-inoculated in 2 mice, but CMAT test performed 4 weeks post-inoculation on sub-inoculated mice was negative.

II.3.3. Bacterial identification

Unfortunately, a lot of mice died only 24-48 hours post-inoculation. This early mortality evoked a bacterial contamination. Digestion pellets samples were randomly selected from each province to verify the presence of bacteria and further to identify bacteria species: 2 chickens from Saraburi, 2 chickens from Lopburi and 1 duck from Nakhonratchasima. The results of bacteria identification after mass spectrometry revealed 8 species of bacteria as shown in Table 30.

Table 30: Bacteria identification from mass spectrometry.

Samples	Location	Species of bacteria
C10 chicken	Saraburi	*Proteus mirabilis*
		Enterococcus faecalis
C12 chciken	Saraburi	*Not identified*
C18 chicken	Saraburi	*Proteus mirabilis*
		Enterococcus faecalis
		Aeromonas hydrophila/sobria
		Morganella morganii
C23 chicken	Lopburi	*Aeromonas hydrophila/caviae*
C26 chicken	Lopburi	*Proteus mirabilis*
		Enterococcus faecalis
		Myroides
D3 duck	Nakhonratchasima	*Escherichia coli*
		Proteus mirabilis

III. Summary of positive DNA from PCR 529 bp based on 3 field works (2014, 2015 and 2016)

Despite all isolation attempts and genetic characterization, we had no sample giving some indications on genotype of *T. gondii* (Table 31) circulating in Thailand. If we exclude the 23 positive DNA detection from 2015 field work that were demonstrated to be due to a

contamination by RH strain, direct DNA detection was positive for 13 tissues digestion pellets (5/65 in 2014, 8/74 in 2016). *Toxoplasma* DNA was detected in tissues (brains and/or hearts) of mice inoculated with 16/162 digestion pellets but no strain was isolated.

Table 31: Summary table of positive DNA and genetic characterization for 3 field works.

Samples	**Positive serological test (Chicken)**	**PCR 529 bp (Ct)**	**15 microsatellite markers**
2014 digestion pellets			
W74	IMAT	39.2/-	No genotype
W75	IMAT	39.5/-	No genotype
W133	IFAT	38.9/39.7	No genotype
W143	IFAT	38.4/-	No genotype
K59	IFAT	40.9/-	No genotype
2016 digestion pellets			
P1 pigeon	NA	41.3/-	No genotype
P3 pigeon	NA	39.2/-	No genotype
Q10 quail	NA	44.7/-	No genotype
C20 chicken	NA	49.9/-	No genotype
C28 chicken	NA	34.9/-	No genotype
C35 chicken	NA	46.9/-	No genotype
C42 chicken	NA	34.3/-	No genotype
C51 chicken	NA	39.9/-	No genotype
Total 13 positive DNA from digestion pellets			
2014 mouse brains			
W97 M2	IMAT	41.5/-	No genotype
W114 M1	IMAT/IFAT	42.2/43.2	No genotype
W117 M2	IFAT	40.5/-	No genotype
2015 mouse brains			
K5 M2	IFAT	38.4/-	No genotype
K7 M2	IFAT	38.3/-	No genotype
K23 M2	IFAT	37.9/40.4	No genotype
K76 M3	IFAT	38.8/-	No genotype
K95 M2	IMAT	39.3/-	No genotype
K121 M3	IMAT	38.1/39.5	No genotype
K129 M3	IMAT	45.0/-	No genotype
W118 M2	IMAT	38.3/-	No genotype
W126 M3	IMAT	35.7/36.8	No genotype
W132 M1	IMAT	45.9/-	No genotype
2016 mouse brains			
C7 chicken	NA	35.4/37.1	No genotype
Total 14 positive DNA from mouse brains			
2014 mouse hearts			
W110 M1	IMAT	42.3/-	No genotype
W114 M1	IMAT/IFAT	29.0/28.9	RH strain (contamination)
W114 M2	IMAT/IFAT	40.3/-	No genotype
2015 mouse heart			
K6 M3	IFAT	48.8/-	No genotype
K129 M2	IMAT	41.8/45.4	No genotype
W126 M3	IMAT	33.8/34.2	No genotype
2016 mouse heart			
C7 chicken	NA	32.9/32.7	No genotype
Total 7 positive DNA from mouse hearts			
Total 34 positive DNA			

Chapter VI: Discussion, conclusion and perspective

I. Sero-epidemiology

Seroprevalence in free-range chickens from diverse parts of Thailand indicated soil contamination by this parasite in rural areas of Thailand. The seroprevalence of *T. gondii* is known to be considerably lower in indoor kept animals than in outdoor kept animals. Free-range chickens are considered as outdoor kept animals. They are widely consumed by Thai population. In rural areas of Thailand, free-range chickens are raised in many families. They are generally kept in the backyard of their houses, where chickens can move and feed freely on the ground (Choprakarn and Wongpichet, 2007).

I.1. Seroprevalence rates vary according to the technique used

In our study, two serological techniques were used, MAT and IFAT. The modified-agglutination-test (MAT) was carried out with 2 different sources of antigen, commercial (CMAT, provided in the Toxo-screen® DA kit) and in-house (IMAT) antigens. According to the technique and the antigen, different seroprevalence rates were obtained. The seroprevalence by IMAT and CMAT was not significantly different (11.5% and 17.7%), whereas the seroprevalence determined by IFAT was significantly higher than seroprevalence obtained by IMAT and CMAT (33.0%). The MAT technique has been validated in chickens (Dubey et al., 2015), whereas the information concerning the accuracy of IFAT is limited, according to (Dubey, 2010b). Our statistical analyses showed a moderate concordance between IMAT and CMAT (0.47), while no substantial concordance was observed between IFAT and CMAT (0.09) and between IFAT and IMAT (0.02).

One factor that may be involved in the *T. gondii* seroprevalence rate is the cutoff of serological tests. Samples tested with IMAT and CMAT to detect anti-*T. gondii* antibodies must be diluted in PBS buffer containing 0.2 mol/L of 2-mercaptoethanol. According to the literature, several positivity cutoff (thresholds) have been reported for MAT test: 1:5 (Dubey et al., 2015), 1:10 (Aroussi et al., 2015), 1:20 (Ayinmode and Olaosebikan, 2014) and 1:25 (Beltrame et al., 2014). The selection of cutoff was difficult. We decided to take 1:10 as cutoff for IMAT and CMAT to avoid possible false positive with the dilution 1:5. If we take 1:20 or 1:25 as cutoff, we can lose some positive chickens at low dilution as 1:10. Dubey et al. (2015) when comparing mouse bioassay results and MAT titers from 19 studies representing a total of 2066 chickens found an isolation rate of only 15.2% for titer 1:5, and 11·4% for titer 1:10.

The selection of IFAT test cutoff was also difficult. Several cutoff have been mentioned by the literature: 1:16 (Casartelli-Alves et al., 2014; Chumpolbanchorn et al., 2009; Sousa et al., 2016), 1:25 (Moré et al., 2012) and 1:40 (Bártová et al., 2009). We decided to follow the majority of previous studies whereby 1:16 was chosen as cutoff in order to avoid the loss of seropositive chickens at low dilutions.

Although MAT is one of the most commonly used test for detection of antibodies against *T gondii* in various species of animals, there is no reference test for *T. gondii* serology. Our statistical analyses to determine the concordance between 3 serological tests showed there was a moderate concordance between IMAT and CMAT. Seroprevalence rate was higher with CMAT (17.7%) than with IMAT (11.5%). The only one difference of IMAT and CMAT is the antigen. The antigen used for IMAT was an in house antigen, produced by our Laboratory of Parasitology, Limoges and this in house antigen was further brought to Thailand by plane. Transportation time and storage conditions may decrease the sensitivity of in house antigen comparing to commercial antigen of CMAT. This showed that the origin and conditions of storage of MAT antigen may also play a role in the differences of seroprevalence reported by diverse studies. Here, we decided to perform CMAT in order to control IMAT and our seroprevalence results showed that CMAT was more sensitive than IMAT. We considered taking CMAT for the subsequent discussion.

Apart from MAT, the common serological techniques used are ELISA, IHAT, IFAT and LAT according to the feasibility and the availability in the laboratory. Different serological techniques used played an important role in the variation of *T. gondii* antibody detection both in humans and in animals. Despite numerous attempts on serological test comparisons, it seems difficult to define the best serological test for *T. gondii* antibody detection in chickens.

Our results showed a higher seroprevalence in free-range chickens by IFAT. In the absence of a gold standard in our study, it cannot be concluded if IFAT gave false positive results or if MAT gave false negative results. Casartelli-Alves et al., (2014) comparing the sensitivity and specificity of serological tests in chickens with bioassay in mice found 48.0% of false positive rate for IFAT and 32.0% of false positive rate by MAT. This may suggest an insufficient sensitivity of bioassay, but also that there is no reliable serological technique. In our study, there was no substantial concordance observed between IFAT/CMAT. However, there was a perfect agreement between MAT and IFAT in other animals as cats and dogs (Macri et al., 2009). A study of seroprevalence in pigs using MAT and IFAT showed 95.7% of sensitivity and 97.8% of specificity in both techniques (Minho et al., 2004). Comparing

these studies with our results, this suggested lack of accuracy for both tests in chickens. During our stay in Mahidol university, an experimental work with chickens intraperitoneally inoculated with *T. gondii* RH strain tachyzoites was conducted to assess the accuracy of IMAT and IFAT for detecting early infection (Chapter personal works III.2.2. and chapter results I.2.). Both tests were able to detect *T. gondii* antibodies in all inoculated chickens. IFAT detected *Toxoplasma* IgG antibody with the titer of 1:32 at the earliest on day 7 in one chicken even in the lower inoculated concentration group (Group 1 = 10,000 tachyzoites/chicken), while IMAT was unable to detect any. One chicken, in higher inoculated concentration group at 40,000 tachyzoites (Group 2 = 40,000 tachyzoites/chicken), was seropositive at the titer of 1:256 by IFAT and 1:100 by IMAT at day 7. This suggested that IFAT was more sensitive than IMAT, but it does not evaluate its specificity in field conditions. The results from the present study bear some resemblance to other experimental chicken studies, who demonstrated that IFAT was able to detect *T. gondii* antibodies in chickens at first to second week post-inoculation, when infected with 1.5 x 10^7 *T. gondii* tachyzoites (RH strain) injected muscularly (Chumpolbanchorn et al., 2009).

The gold standard for *T. gondii* infection is isolation of *Toxoplasma* DNA in tissues or isolation of viable strains. In our study, in digestion pellets from brain and heart of positive chickens, the detection rate of *T. gondii* DNA was quite low, indicating a low amount of parasites even though the chickens were positive by serological tests. Nevertheless, it is difficult to interpret our uncorrelated results between the serological tests, *T. gondii* DNA detection and *T. gondii* strain isolation.

I.2. The low seroprevalence obtained in chicken from Kanchanaburi is in agreement with other studies in Asia

A previous seroprevalence study on free-range chickens in suburb of Bangkok by using IFAT found a high seroprevalence (64.03%) (Chumpolbanchorn et al., 2009), higher than in our study (33.0% with the same technique). Most of their free-range chickens were older than 5 months, whereas our free-range chickens were between 2-5 months. This may partly explain the difference in seroprevalence rates between these 2 Thai studies using the same serological technique. The infection with *T. gondii* increases with age together with the risk of exposure to risk factors. In our study, we did not find any age effect on seroprevalence rates (2-3 month old compared to 4-5 month old chickens) but this may be explained by a relative uncertainty of age estimation by farmers.

The seroprevalence rates obtained by CMAT in our study is in the range of seroprevalence rates in free-range chickens in diverse parts of Asia: 11.4% (MAT) in southern China (Yan et al., 2009), 5.8% (MAT) in northeastern China (Yang et al., 2012), 24.4% and 24.2% (MAT) in Indonesia and Vietnam respectively (Dubey et al., 2008). Seroprevalence of *T. gondii* infection in Asian free-range chickens appeared relatively low when compared with the high seroprevalence in free-range chickens in South America and Africa: 65.5% in Argentina, 65.1% in Brazil, 85.7% in Nicaragua, 40.4% in Egypt and 64.0% in Ghana (reviewed in Dubey (2010b). Seroprevalence with MAT in Asian free-range chickens is more similar to seroprevalence in free-range chickens in European and North American studies: 13.7% in Italy (Dubey et al., 2008), 16.9% in Ohio, USA (Dubey, 2010b). This low seroprevalence in Asian chickens may reflect a lower contamination of environment with *T. gondii* oocysts than in South America or tropical Africa. Felids are the main reservoirs of *T. gondii*, they are the only animals which are able to excrete oocysts that contaminate the environment. Numerous studies on seroprevalence in felids from South America or Africa, showed high seroprevalence in domestic and feral cats, up to 87.3% in Brazil (reviewed by Bolais et al., 2017) or 95.5% in Egyptian cats (Al-Kappany et al., 2011). Seroprevalence data in felids from tropical Asia are scarce and used different techniques, but seem to indicate a low rate of infection. Only 4.8% of cats in Thailand were seropositive to *T. gondii* using Sabin Feldman Dye Test (Jittapalapong et al., 2010), 7.3% of cats in Bangkok, Thailand using Sabin Feldman Dye Test (Sukthana et al., 2003) and 11.0% of stray cats by LAT (Jittapalapong et al., 2007). The seroprevalence of cats in Malaysia was 14.5% by IFAT (Chandrawathani et al., 2008). This is in agreement with the low seroprevalence (only 6.4% using Sabin-Feldman Dye Test) of persons having close contact with cats as cat owners in Thailand, (Sukthana et al., 2003). In Malaysia, the seroprevalence of *T. gondii* in people having close contact with animals such as veterinarians, technicians, students and pet owners was 19.9% using ELISA (Brandong-Mong et al., 2015). The fact that domestic Thai cats are mostly fed on rice and fish or well cooked meat may partly explained the low seroprevalence in cats, and hence the low environmental contamination of soils and water (Sukthana, 2006). This may not be the case in free-range cats. This low environmental contamination may result in a low rate of infection in free-roaming chickens as in our study or in other intermediate hosts in Thailand: 9.4% in cows by LAT (Inpankaew et al., 2010), 10.9% in stray dogs by LAT (Jittapalapong et al., 2009), only 4.6% in rodents by LAT (Jittapalapong et al., 2011).

II. Detection of *T. gondii* DNA, isolation and factors affecting the isolation

The bioassay in mice or cats is the classic gold standard method to evaluate the presence of *T. gondii* in animal tissues. However *Toxoplasma* DNA detection is now also considered as a substitute or complement to this gold standard when isolation turned out to be impossible.

Overall, 162 isolation attempts were performed by bioassays in mice but no viable strain was isolated. During the first and third field works, the majority of mice died 24-48 hours post-inoculation. DNA extracts from mouse brains or hearts from the remaining mice were positive in 8/94 with low amount of *T. gondii* DNA, but microscopic examination was negative. For the second field work, all mice survived and 71/216, (32.9%) were seropositive by IMAT test. Microscopic examination did not find any *T. gondii* cysts, and sub-inoculations of frozen brains of these 71 seropositive mice were negative although 10/71 (14.1%) mouse brain DNA and 3/71 (4.2%) mouse heart DNA were slightly positive by PCR 529 bp (Ct values > 33). These negative microscopic examinations and sub-inoculations lead us to consider these PCR results on mouse tissues as possible false positive.

During our 3 field works, direct *Toxoplasma* DNA detection on DNA extracts from digestion pellets (brain and heart of seropositive animals) with a real time PCR 529 bp was attempted on 211 samples. It was positive in 36 cases. Unfortunately, 23 of these 36 positive DNA extracts, corresponding to the second field work, were demonstrated by MS genotyping to be due to DNA contamination by the laboratory RH strain type 1 used in the laboratory. So, when excluding results from this second field work, 13/139 DNA extracts were positive for *T. gondii* DNA.

Analysis of the diverse problems encountered during this work

The different problems encountered during our thesis work lead us to discuss about all the possible causes leading either to false-positive results (DNA contamination, positive serology in mice without strain isolation) or to the absence of strain isolation (conditions and organization of field work, bacterial contamination).

DNA contamination

DNA contamination by RH strain was demonstrated for all the positive digestion pellets obtained in 2015 and for one positive sample (mouse heart) in 2014. The demonstration of this contamination was made possible thanks to the high discriminatory power of MS genotyping (0.99) (Ajzenberg et al., 2010). RH strain or DNA extracted from

suspensions of RH tachyzoites is used in many laboratories as positive controls for PCR assays used to detect DNA in animal studies. It is extremely easy to get contamination even in strictly controlled environments due to the large amount of RH strain DNA manipulated during these assays. This may lead to positive results reported in the literature, which may, actually, be false positive results especially in studies using nested-PCR (Ajzenberg, 2010). In some cases, genotyping of these positive DNA extracts are performed and type I strains are identified. But the usual genotyping method, PCR-RFLP, is not able to differentiate the RH strain from other type I strain, due to a lack of discriminatory power. This can cast a doubt on papers reporting a majority of type I or reporting a high rate of positive DNA detection in their samples.

Finding the origin of a contamination is not easy. In our case, it could have occurred for the 2014 field work during the extraction step on mouse heart which was not performed in a dedicated room of the laboratory. Contamination of an automatic pipette was suspected for DNA detection in digestion pellet extract from 2015: a *Toxoplasma* PCR performed on DNA extracted from swabs passed on this automatic pipette was positive. For our DNA detection assays, the same lab bench was used to add our samples and the positive and negative controls in the tubes. Our samples may have been contaminated during this step. We decided to separate the lab bench for the samples and the positive and negative control to avoid having the contamination. The manipulation with positive control should be more careful in order to avoid the problem of contamination. All materials (lab bench, centrifuges, automatic pipettes and lab hood) were decontaminated according to the procedure of Laboratory of Parasitology, Limoges and were controlled by PCR 529 bp to verify that they were not contaminated.

Positive serology in mice without strain isolation

During the second field work, 4 weeks post-inoculation, an IMAT serology carried out for all 216 inoculated mice was positive for 71 of them (Table 26). Even with MAT titers as high as 1:800 (34/71 mice), no strain could be isolated from these seropositive mice. The first hypothesis may be technical serology problems, but control sera used in each MAT series were conformed and IMAT is more prone to give false negative than false positive results. Another explanation, which is evoked in the case of blood inoculation, is the inoculation of antibodies together with the sample. It can be excluded in our cases. Finally, a possible explanation may be that mice produce antibodies after injection of dead parasites. Injecting inactivated parasites has already been used for producing antibodies and even for inducing a protective immunity in experimental assays (Jongert et al., 2009). This would suggest that pretreatment of our samples killed the parasites. During our first field work, we carried out

pepsin digestion of tissue which is the method commonly recommended by Dubey (Dubey, 2010a). As we did not succeed in isolating strains, we hypothesized that chicken tissues may contain tachyzoites which are known to be destroyed by pepsin. During our second field work, we changed for a trypsin digestion, as tachyzoites are resistant to trypsin. The trypsin digestion procedure is classically carried out in our laboratory with a good rate of strain isolation. From our experience, we have no clear explanation for a possible death of all the parasites after trypsin digestion. The sub-inoculation was performed with aliquots of seropositive frozen mouse brains (kept at -80°C and then -196°C) and the results were negative for all sub-inoculated mice. The viability of live parasite may decrease with the freezing time. If our fresh samples contained low parasite, it is possible that the number of parasites decreases even more with freezing.

Presence of *Toxoplasma* DNA in mouse brain without strain isolation

Fourteen mouse brains were positive for *Toxoplasma* DNA detection but live parasites were not isolated. If we exclude the possibility of a DNA contamination, an explanation for this non-isolation may be the very low number of *Toxoplasma* in our initial samples. When aliquots of corresponding frozen mouse brains were used for new mice, these sub-inoculating did not develop a *Toxoplasma* infection. Freezing is associated with a partial loss of viability and, in case of a low number of parasites, this may result in an absence of infection. It may be proposed to use immunosuppressed mice for the sub-inoculation from frozen mouse brain, as the development of parasite should be favored by the alteration of their immune system (Puvanesuaran et al., 2012).

Conditions of field work

- **Temperature**

Thailand is a tropical country. The temperature in winter season is between 10-23 °C and the temperature in summer season is between 28-39 °C. The temperature may exceed 40 C in the hottest season (April-August). Regarding our fieldwork periods, our project was carried out during April to August, which is the hottest period of Thailand. The high temperature can favor the development and multiplication of numerous bacteria in our fresh samples before putting them into the ice boxes for transportation, especially for samples collected on the markets.

- **Transportation time and processing delays**

Fresh samples (brain and heart of positive animals) were stored in ice boxes during the sample collection and the transportation time. The duration of the transportation time varied according to the road traffic. The duration from Kanchanaburi to Bangkok is generally about 3-4 hours but we were confronted with the traffic jam in the center of Bangkok, which increases the transportation time. In some cases, the ice melted, resulting in an increase of sample storage temperature. During the third sample collection, our samples were successively transported by the farmers from the farms to the market, and then by several types of transport as car, train and plane from the markets to Limoges laboratory. These transportation delays could decrease the viability of *T. gondii* in the samples. Dubey et al., (2008) described the low isolation rate of free-range chickens from Ghana, Indonesia and Vietnam, which may have resulted from the delay in transport that provoked the lysis of tissues.

Besides the transportation time, there were additional delays for sample processing. Eight to sixteen isolation attempts were carried out each day, leading to nearly a week of supplementary waiting period for the last processed samples. A study of *T. gondii* bradyzoite viability was conducted in our laboratory by a Master student (Radouane M.). to evaluate the viability of *T. gondii* bradyzoites at 4°C and 22°C for 18 days (Figure 35). High mortality rate was observed at 22°C increasing with the number of observation days. At 4°C, the mortality rate increased at day 8. This viability experimentation shows that, in our case, a loss of viability could partially explain the non-isolation for this third field work. Our fresh samples were retrieved and kept at 4°C 1-10 days in Thailand before taking to Limoges, France and we take into account the beginning of isolation process, some samples could have been treated up to 17 days after the death of the animal.

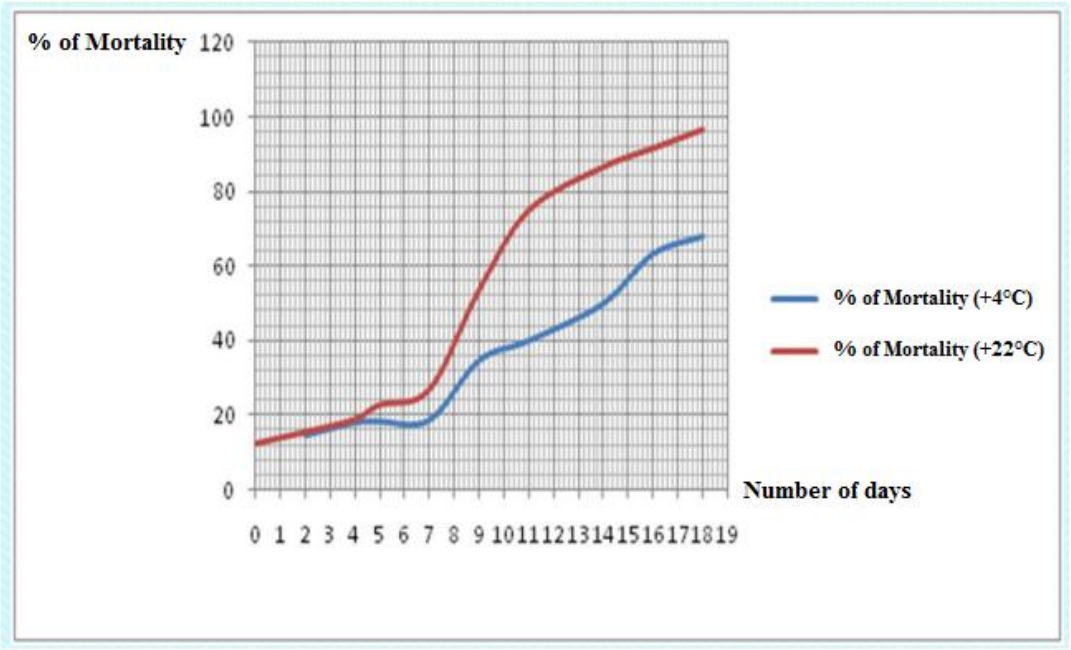

Figure 35: Viability of *T. gondii* bradyzoites in NaCl 0.9% (modified from Radouane M. Master Limoges, 2010 « Techniques d'évaluation de la viabilité des kystes de *Toxoplasma gondii* après congélation et optimisation du protocole de congélation »).

- **Early mouse mortality**

In case of virulent strains, mice died within 7 to 15 days with tachyzoites in the intraperitoneal fluid or in other tissues, notably lungs. In our study, we observed a high rate of mortality 24-48 hours post inoculation. Such a short delay of death suggested a bacterial infection rather than an infection by a virulent strain. The hypothesis of a vascular lesion secondary to inoculation was also ruled out, as it would have provoked a death quasi-immediately after inoculation.

- **Bacterial contamination**

The tropical climate of Thailand increases the risk of exposure to certain microorganism as bacteria especially in the hot season, where the temperature may exceed 40 °C. Moisture and heat induce the rapid multiplication of various microorganisms including bacteria. After our first mouse inoculation experiment, we observed the death of 119/148 (80.4%) dead mice only 24-48 hours post-inoculation. Streptomycin (10mg/ml) and penicillin (10,000U) were used for this first isolation attempt. We decided to replace streptomycin and penicillin by cefotaxime (1g/10ml), vancomycin (500mg/10ml) and ciprofloxacin (200mg/100ml) according to the experience of our colleagues in Reims (D. Aubert, personal communication). Despite the modification of antibiotic regimen, the problem of mouse

mortality remains unresolved. An identification of bacteria was carried out on randomly selected samples collected in the different markets to search for a possible resistance of these bacteria to antibiotics. *Proteus mirabilis, Enterococcus faecalis, Aeromonas hydrophila/sobria/caviae, Morganella morganii, Myroides and Escherichia coli* were found in our samples. *Proteus mirabilis* is an opportunistic pathogen and it was reported that the reservoir of *P. mirabilis* is intestine in several species as mammals, birds, reptiles, amphibians, insects, and seafood. Soil and water could be contaminated by *P. mirabilis* by the fecal pollution (Drzewiecka, 2016). The most common species of *Enterococcus* is *Enterococcus faecalis.* It is very resistant to pH and temperature variations (E et al., 2015). The majority of *E. faecalis* was found in gastrointestinal tracts and feces of mammals, reptiles, birds and insects. Fecal contamination leads to the contamination of plants, soil and water by *E. faecalis* (Lebreton et al., 2014). *Aeromonas hydrophila, A. sobria, A. caviae* are ubiquitous in ecosystem. They can be found in aquatic habitats, fish, foods, domesticated pets, invertebrate species, birds, ticks, insects, and natural soils. Several of them were isolated from water and aquatic environments such as rivers, lakes, seawater, drinking water, ground water and wastewater (Janda and Abbott, 2010). A study was conducted in Thailand after the Tsunami disaster in December 2004. *Aeromonas* species were the major organisms isolated (22.6%) from the survivors. This study suggested that these survivors should be exposed to the contaminated water from the flooded areas (Hiransuthikul et al., 2005). *Morganella morganii* is commonly found in intestinal tracts of humans, reptiles, mammals and also in environment and occurs in a little number in feces of humans and animals (Liu et al., 2016). *Myroides* is an ubiquitous organism. It has been widely isolated from various sources such as food and especially in water (Hugo et al., 2006). They are resistant to several common antibiotics as penicillin, cephalosporin, aminoglycoside, quinolone and carbapenems (Elantamilan et al., 2015). *Escherichia coli* is widely presented in gastrointestinal tract of humans and animals. This species is widespread throughout the world and can contaminate the environment with the fecal transmission (Caprioli et al., 2005). These bacteria could provoke the sepsis in mice, which is the cause of rapid mortality.

Furthermore, these bacteria are resistant to the antibiotics we used (Table 32). This is a major problem in isolation especially in bioassay in mice. Several hypotheses may explain the contamination of our samples by these bacterial species. As described above, fecal pollution and contaminated environments as soil, plant and water are the main sources of transmission of these bacteria. Our samples were collected from several fresh markets in Thailand. It is possible that the chicken owners did not wash their hands when they dissected their chickens

to retrieve the internal organs. In addition, most of fresh markets in Thailand are open air markets, without air-conditioning, this may favor the bacterial proliferation by airstream.

Ciprofloxacin was the most efficient antibiotic among 6 antibiotics used in our experimentation. The resistance to ciprofloxacin was found only for *Morganella morganii*. Gentamicin and vancomycin were the least efficient antibiotics, only *Myroides* was sensitive to gentamycin and only *Enterococcus faecalis* was sensitive to vancomycin. Penicillin had the effect on 2 bacterial species: *Enterococcus faecalis* and *Myroides*. Cefotaxime had also effect on 2 bacterial species: *Proteus mirabilis* and *Morganella morganii*. The antibiogram of streptomycin was performed only for *Enterococcus faecalis* and showed that this species was sensitive to streptomycin. If we take into account this antibiogram results, a new combination of antibiotics including ciprofloxacin (maximum concentration 3mg/ml, without exceeding 30 mg per day for subcutaneous injection in mice) (Bourgeois et al., 2016) may be proposed to avoid a bacterial invasion.

Table 32: Antibiogram showing the resistance and the sensitivity to antibiotics of bacteria isolated from chicken samples collected on the Thai markets.

Bacteria species	Samples	Ampicillin/ Penicillin	Gentamicin	Streptomycin	Cefotaxime	Vancomycin	Ciprofloxacin
Proteus mirabilis	C10, C18, C26, D3	Resistant	Resistant	-	Sensitive	Resistant	Sensitive
Enterococcus faecalis	C10, C18, C26	Sensitive	Resistant	Sensitive	Resistant	Sensitive	-
Aeromonas hydrophila/sobria/caviae	C18, C23	Resistant	Resistant	-	Resistant	Resistant	Sensitive
Morganella morganii	C18	Resistant	Resistant	-	Sensitive	Resistant	Resistant
Myroides	C26	Sensitive	Sensitive	-	Resistant	Resistant	Sensitive
Escherichia coli	D3	Resistant	Resistant	-	Resistant	Resistant	Sensitive

Organization of field work

This thesis work is the first project, which focused on the isolation of *T. gondii* strains in free-range chickens and other dead animals found on the road in Thailand. There are some studies concerning *T. gondii* seroprevalence in humans and animals but no *T. gondii* isolation study was performed in this area. Although we tried to control other factors such as the temperature and bacterial contamination, by using ice boxes, by adding antibiotics or by shortening the delay of transportation, we did not succeed in isolating strains. Sometimes, we had to collect many fresh samples per day (10-20 samples) according to the availability of the

veterinary. This resulted in an intensive laboratory experimentations leading to an increased delay of treatment for some samples.

Conclusion and perspective

In conclusion, this work demonstrated the importance and difficulties of field work for the seroprevalence study as well as the isolation study. Our seroprevalence results in free-range chickens showed that *T. gondii* is circulating in the Thai rural environment. Outdoor kept animals such as free-range chickens have a high risk to be infected with *T. gondii* oocysts. The isolation of *T. gondii* strains required an intensive sampling field work, which is complex in a tropical and humid environment as found in Thailand. Many parameters could have a negative impact on our work and the result is not guaranteed because the seropositive animals did not allow isolating the strains, possibly due to a low quantity of parasite. Furthermore, there is a lack of sensitivity and specificity of the serological tests. It is indispensable to find the reliable serological test in order to detect the true positive animals. As our project is the first isolation work in Thailand, many adjustments regarding the organization and the preparation of field works as well as laboratory experimentations are needed for a better future isolation project (Annex 2). The preparation of official documents is indispensable such as animal ethic document, authorization to import biological samples. The temperature is an important factor for the live parasite isolation. High temperature decreases the viability of live parasite and supports the multiplication of microorganisms in fresh samples. The use of well-adapted antibiotics is necessary to reduce the problem of bacterial contamination, due to the possibility of circulation of resistant bacteria.

Publications and communications

I. Publications related to the thesis

1. Udonsom*, R., **Chaichan*, P.**, Mahittikorn, A., Vignoles, P., Mercier, A., Aroussi, A., Dardé, M.L., Sukthana, Y. *Toxoplasma* serostatus in Thai free-range chickens: prevalence and two diagnostic methods. Submitted version.

2. **Chaichan, P.**, Mercier, A., Galal, L., Mahittikorn, A., Morand, S., Boumédiène, F., Udonsom, R., Hamidovic, A., Murat, JB., Sukthana, Y., Dardé, M.L. Geographical distribution of *Toxoplasma gondii* genotypes in Asia: a link with neighboring continents. Accepted version.

II. Scientific poster

1. Udonsom, R., Mahittikorn, A., **Chaichan, P.**, Sukthana, Y. Comparison of toxoplasmosis diagnosis by indirect fluorescent antibody test and modified agglutination test in free-range chicken in Thailand. **Presented in** Joint Conference 2014: the 55th Annual Meeting of the Japanese Society of Tropical Medicine and the 29th Annual Meeting of the Japan Association for International Health: 1-3 November 2014.

2. Ganteil, A., Bolais, P., **Chaichan, P.,** Ajzenberg, D., Dardé, M.L., Mercier, A. Etude de la dynamique de transmission du parasite *Toxoplasma gondii* dans une ferme en Limousin (Lieu-dit Villemonteix, Glanges). **Presented in** Journée GEIST - Limoges 4 September 2015.

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Annex

Annex 1: Felidae

More recently, there are 37 species in the Felidae family worldwide (Figure 36), which distributed in 8 lineages (O'Brien and Johnson, 2007).

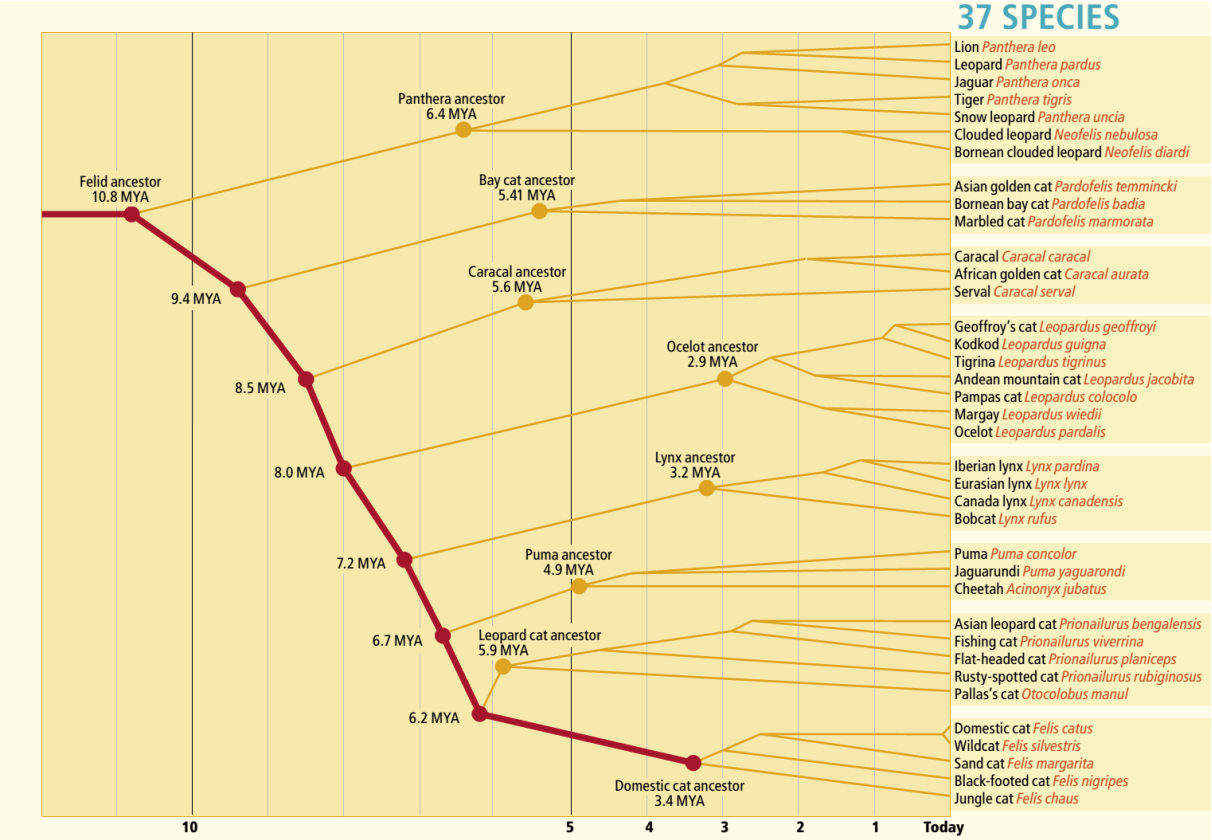


Figure 36: Felidae family tree (O'Brien and Johnson, 2007)

The most remarkable scenario involves that the modern felids emerge in Asia with the divergence of the *Panthera* lineage 10.8 MYA and subsequently, the bay cat lineage 9.4 MYA (Johnson et al., 2006).

The Felidae species shown in the table below are the species have been found in Southeast Asian countries (O'Brien et al., 2008).

I. Wild felids in Southeast Asian countries

I.1. Tigers (*Panthera tigris*)

The tiger is one of the best-known mammals. Their yellow color with black stripes is immediately recognizable. There are 8 subspecies of tigers with 3 subspecies are nowadays extinct, Bali tiger, Javan tiger and Caspian tiger (http://www.bigcatswildcats.com).

- Indochinese tiger (*Panthera t. corbetti*)
- Bengal tiger (*Panthera t. tigris*)
- Sumatran tiger (*Panthera t. sumatrae*)
- Malayan tiger (*Panthera t. jacksoni*)
- South China tiger (*Panthera t. amoyensis*)
- Bali tiger (*Panthera t. balica*)
- Javan tiger (*Panthera t. sondaica*)
- Caspian tiger (*Panthera t. virgata*)

The tigers are found in a variety of habitats such as, tropical evergreen forests, deciduous forests, mountains, birch woodlands, etc. Tigers usually hunt and attack their prey with a small from the rear. Currently, the number of tigers has decreased. There are approximately 7,700 tigers in the world, there may have been 100,000 tigers at the end of the 19th century (Macdonald et al., 2010).

I.2. Bornean bay cat (*Catopuma badia*)

The Bornean bay cat has 2 colors, chesnut-red and grey. It is a rare subspecies. The bay cat resembles to the Asiatic golden cat. They live near rivers, forests and as well as the highlands. The bay cat only occurs on the island of Borneo which comprises 3 countries, Brunei, Indonesia and Malaysia). The Bornean bay cat is also one of the most endangered wild cat species. The Bornean bay cats have been considered rare and their number is insufficiently known (Macdonald et al., 2010).

I.3. Clouded leopard (*Neofelis nebulosa*)

Clouded leopard is the species of wild cat that live throughout the forests of Southeast Asia. The clouded leopard has arboreal talents, they can run down tree trunks headfirst, climb about on horizontal branches with its back to the ground, and hang upside down from branches by its hind feet. The clouded leopard habitats are mostly associated with the

evergreen tropical rain forest but can also be found in dry woodlands and secondary forests. Same as all wild cats, clouded leopards are carnivorous; they hunt a variety of prey including birds, squirrels, monkeys, deer and wild pigs. There is no data on population status of Clouded leopard (Macdonald et al., 2010).

I.4. Asiatic Golden Cat (*Catopuma temmincki*)

They can be red-golden, brown, black or grey in color for their pelage. Asiatic Golden Cats are able to climb the trees. They are found in sub-tropical and tropical humid evergreen and dry deciduous forests. Asiatic Golden Cats are carnivorous and they feed on small deer, rodents, birds, amphibians, lizards and insects. They are reported as uncommon and threatened by deforestation (Macdonald et al., 2010).

I.5. Flat-headed cat (*Prionailurus planiceps)*

The Flat-headed Cat takes its name from its unusually long, sloping snout and flattened skull roof. They have the thick and soft pelage with the red-brown color tinged with grey. They live in wetland and lowland forests. They eat normally fish, shrimp and domestic poultry. Their population status is insufficiently known (Nowell K. and Jackson P., 1996).

I.6. Fishing cat (*Prionailurus viverrinus*)

Fishing cats have the powerful short limbs and a stocky body. They have a long head and a short tail. The color of their pelage is often brown and grey. The size of fishing cats varies with gender. Their habitats are highly associated with the wetlands both swamp and marshy areas. They scoop their prey from the depth of the water. The information regarding their population status is also insufficiently known (Macdonald et al., 2010).

I.7. Marbled cat (*Pardofelis marmorata*)

They resemble a small Clouded leopard. The marbled cat's pelage is similar to that of clouded leopard. The color of the pelage is brown-grey through red-brown. Their fur is thick and soft with a well-developed underfur. They usually live in the moist tropical forest and also in evergreen and deciduous forests. Marbled cats climb and hunt in trees for arboreal mammals for example squirrels, as well as birds. They may be a naturally rare species but their population status is not adequately known (Macdonald et al., 2010).

I.8. Leopard (*Panthera pardus*)

The leopard has relatively short legs and long body. It most closely resembles to the jaguar. The leopard is known for its ability in climbing and it has been observed that leopard can stay on tree branches during the day. They are the powerful swimmer and they are able to take large prey. Leopards are frequent in tropical regions in Asia. Leopard may have increased throughout the region (Macdonald et al., 2010).

I.9. Jungle cat (*Felis chaus*)

The jungle cat is a small-medium cat. The important characteristic of a jungle cat are equal-sized claws on both front and hind legs which allow them to climb down trees. They normally feed on rodents. They are not closely associated with the forests but rather associated with water and dense vegetative cover. Jungle cats can adapt to many different types of agricultural and forest plantations. The species is considered common (Macdonald et al., 2010).

I.10. Leopard cat (*Prionailurus bengalensis*)

The size of leopard cats is similar to the size of domestic cats. There is a good deal of variation in their pelage. The body and limbs are marked with black spots of different sizes and colors. Leopard cats are excellent swimmers. Some of them are active during the day but mostly hunt at night. There is a wide distribution of leopard cats in Asia and in Southeast Asia. They live in tropical rainforests, coniferous forests and as well as in the grasslands. The principle foods of leopard cats are rodents and other small mammals. They are common and may have been eliminated by deforestation (Macdonald et al., 2010).

II. Domestic cats (*Felis catus*) in Southeast Asian countries

Domestic cats are worldwide animals including in Southeast Asia. Housecats have a high reproduction rate and there are a lot of stray cats in the markets, on the streets, in the temples and several public places. Cats are feeding normally from meat and fish. Domestic cats are the great pest control agents for rodents around areas of human habitation. Domestic cats are widespread and are not obviously in danger (http://www.animaux.org/chat-domestique).

Annex 2: List of materials required for the field work

General materials
1. Freezers -20 à -80 °C
2. Centrifuge (for 250 ml Falcon tubes)
3. Vortex
4. Refrigerators
5. Optical microscopy
6. Pipettes-micropipettes
7. Glove
8. Clips, scissors, syringes and needles
9. Tubes 1.5-2.0 ml
10. GPS
MAT serological test
1. Microtiter plates 96 wells U bottom
2. Suspension of antigen (in house or commercial)
3. BABS buffer
4. PBS buffer
5. 2-mercaptoethanol
6. Parafilm or adhesive sheets
Animal sampling
1. Markers for animals
2. Tubes for animal blood sampling
Tissue digestion
1. Trypsin
2. Pepsin
3. HCl (only for pepsin digestion)
4. NaCl 0.9%
5. Grinder
6. Pads for centrifugation tubes 175 mL to 225 mL
7. Sterile bottles (250 ml) for centrifugation
8. Glass bottles for digestion (250 ml -1L)
9. Water bath with agitation
10.Sodiumbicarbonate (only for pepsin digestion)
11. Gauze
Mouse inoculation
1. Syringes and needles 20 et 23G
2. Mice (Swiss mice - female 20-25g)
3. Penicillin 10000 U and Streptomycin 10 mg/mL (better with new antibiotic as ciprofloxacin)
Treatment of the mouse brain after inoculation
1. Ketamine 50mg/ml or CO_2
2. Syringes and needles 20G
3. Slides and cover-slide
4. Clips and scissors
Mouse Brain freezing
1. RPMI or MEM
2. DMSO 10%
3. BFS Bovine fetal serum
4. Cryotubes 1.5-2.0 ml

5. Minicryoconservator (Biocell) 6. Cryoconservator 7. Liquid Nitrogen – 196°C 8. Freezers – 80°C
DNA extraction
1. QIAGEN DNA mini-kit 2. Minicentrifuge

Annex 3: DNA extraction (Minikit Qiagen®: QIAamp DNA Minikit, Qiagen SA, Courtaboeuf, France).

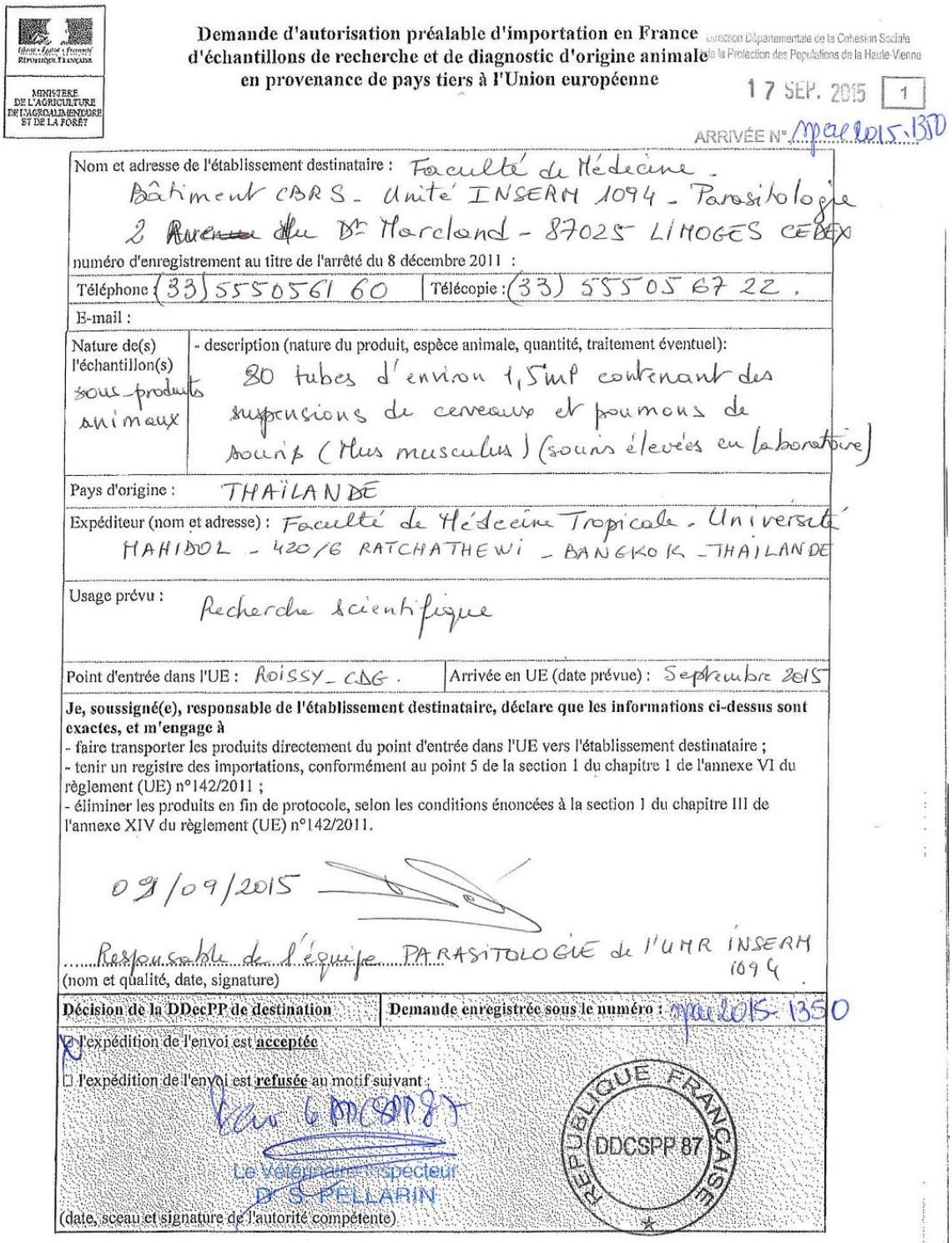
I. DNA extraction from fluids (digestion pellets, mouse brain suspension)

Two hundred µl of sample were placed in 1.5 ml tube. Then 180 µl of AL buffer and 20 µl of proteinase K were added to the sample. This step is intended to lyse all cellular components in order to release the *Toxoplasma* DNA. The sample was incubated 1-3 hours at 56°C. After this incubation, the sample tube was briefly homogenized and 200 µl of ethanol were added to the 1.5 ml tube. The sample was transferred into the QIAamp spin column and centrifuged at 8000 rpm for 1 minute. The eluate was removed, and 500 µl of washing buffer AW1 were added into the column and centrifuged at 8000 rpm for 1 minute. The eluate was removed, and 500 µl of washing buffer AW2 were added into the column and centrifuged at 14000 rpm for 3 minutes. The eluate was discarded and the column was placed on a new 1.5 ml tube then 200 µl of AE buffer was added into the column. The sample was incubated for 5 minutes at room temperature and centrifuged at 8000 rpm for 1 minute. The column was thrown away and 200 µL of eluate were recovered in a 1.5 ml tube. This eluate contained DNA extract and was stored at -20°C until used.

II. DNA extraction from tissues (mouse heart, mouse lung)

The tissues (less than 25 mg) were cut into small pieces and were placed in 1.5 ml tube. One hundred and eighty µl of ATL buffer and 20 µl of proteinase K were added to the sample. The sample was incubated 1-3 hours at 56°C. The lysis overnight is possible. After the incubation, the sample tube was briefly homogenized and then 200 µl of AL buffer were added to the 1.5 ml tube and was incubated at 70°C for 10 minutes. After the second incubation, the sample was mixed by pulse-vortexing for 15 seconds and then 200 µl of ethanol were added to the sample and transferred in the QIAamp spin column. The sample was centrifuged at 8000 rpm for 1 minute. The eluate was removed then 500 µl of washing buffer AW1 was added into the column and centrifuged at 8000 rpm for 1 minute. The eluate was removed then 500 µl of washing buffer AW2 was added into the column and centrifuged at 14000 rpm for 3 minutes. The eluate was discarded and the column was placed on a new 1.5 ml tube then 200 µl of AE buffer was added into the column. The sample was incubated for 5 minutes at room temperature and centrifuged at 8000 rpm for 1 minute. The column was thrown away and 200 ml of eluate were recovered in a 1.5 ml tube. This contained Toxoplasma DNA extract. Toxoplasma DNA was stored at -20 °C until used.

Annex 4: Import authorization



MINISTÈRE DE L'AGRICULTURE DE L'AGROALIMENTAIRE ET DE LA FORÊT

Demande d'autorisation préalable d'importation en France d'échantillons de recherche et de diagnostic d'origine animale en provenance de pays tiers à l'Union européenne

Direction Départementale de la Cohésion Sociale et de la Protection des Populations de la Haute-Vienne

17 SEP. 2015 | 1

ARRIVÉE N° mar2015-1350

Nom et adresse de l'établissement destinataire : Faculté de Médecine. Bâtiment CBRS - Unité INSERM 1094 - Parasitologie 2 Avenue du Dr Marcland - 87025 LIMOGES CEDEX

numéro d'enregistrement au titre de l'arrêté du 8 décembre 2011 :

Téléphone : (33) 5550561 60 | Télécopie : (33) 555056722.

E-mail :

Nature de(s) l'échantillon(s) sous-produits animaux

- description (nature du produit, espèce animale, quantité, traitement éventuel): 80 tubes d'environ 1,5ml contenant des suspensions de cerveaux et poumons de souris (Mus musculus) (souris élevées en laboratoire)

Pays d'origine : THAÏLANDE

Expéditeur (nom et adresse) : Faculté de Médecine Tropicale - Université MAHIDOL - 420/6 RATCHATHEWI - BANGKOK - THAILANDE

Usage prévu : Recherche scientifique

Point d'entrée dans l'UE : Roissy_CDG. | Arrivée en UE (date prévue) : Septembre 2015

Je, soussigné(e), responsable de l'établissement destinataire, déclare que les informations ci-dessus sont exactes, et m'engage à

- faire transporter les produits directement du point d'entrée dans l'UE vers l'établissement destinataire ;
- tenir un registre des importations, conformément au point 5 de la section 1 du chapitre 1 de l'annexe VI du règlement (UE) n°142/2011 ;
- éliminer les produits en fin de protocole, selon les conditions énoncées à la section 1 du chapitre III de l'annexe XIV du règlement (UE) n°142/2011.

09/09/2015

Responsable de l'équipe PARASITOLOGIE de l'UMR INSERM 1094

(nom et qualité, date, signature)

Décision de la DDecPP de destination | **Demande enregistrée sous le numéro :** mar2015-1350

☒ l'expédition de l'envoi est **acceptée**

☐ l'expédition de l'envoi est **refusée** au motif suivant :

Le Vétérinaire Inspecteur
Dr S. PELLARIN

RÉPUBLIQUE FRANÇAISE DDCSPP 87

(date, sceau et signature de l'autorité compétente)

MINISTERE DE L'AGRICULTURE DE L'AGROALIMENTAIRE ET DE LA FORÊT

Demande d'autorisation préalable d'importation en France d'échantillons de recherche et de diagnostic d'origine animale en provenance de pays tiers à l'Union européenne

Direction Départementale de la Cohésion Sociale et de la Protection des Populations de la Haute-Vienne

2 4 MAI 2016

ARRIVÉE N°. ppae2016_0694

Nom et adresse de l'établissement destinataire : Faculté de Médecine
Bâtiment CBRS – Unité INSERM 1094 – Parasitologie
2 rue du Dr Marcland – 87025 Limoges Cedex

numéro d'enregistrement au titre de l'arrêté du 8 décembre 2011 : 2015348-005-ddcspp

Téléphone : +33 (0) 5 19 56 42 65 +33 (0) 6 95 35 55 89	Télécopie : +33 (0) 5 55 43 58 21
E-mail : aurelien.mercier@unilim.fr	
Nature de(s) l'échantillon(s) Sous-produits animaux	- description (nature du produit, espèce animale, quantité, traitement éventuel): 150 tubes contenant 2-3 ml de suspension de cerveaux et de poumons de souris (*Mus musculus*) (souris élevées en laboratoire), 300 tubes contenant 1-2 ml de sérum de poulets (*Gallus domesticus*)
Pays d'origine : THAILANDE	
Expéditeur (nom et adresse) : (Contact : Dr Aongart Mahittikorn) Faculté de Médecine Tropicale – Université MAHIDOL – 420/6 RATCHATHEWI – BANGHOK - THAILANDE	
Usage prévu : Recherche scientifique	
Point d'entrée dans l'UE : ROISSY-CDG	Arrivée en UE (date prévue) : 31/05/2016

Je, soussigné(e), responsable de l'établissement destinataire, déclare que les informations ci-dessus sont exactes, et m'engage à

- faire transporter les produits directement du point d'entrée dans l'UE vers l'établissement destinataire ;
- tenir un registre des importations, conformément au point 5 de la section 1 du chapitre 1 de l'annexe VI du règlement (UE) n°142/2011 ;
- éliminer les produits en fin de protocole, selon les conditions énoncées à la section 1 du chapitre III de l'annexe XIV du règlement (UE) n°142/2011.

Pr Pierre-Marie PREUX, Directeur de l'UMR Inserm 1094.....
(nom et qualité, date, signature)

Décision de la DDecPP de destination | **Demande enregistrée sous le numéro :** ppae2016_0694

X l'expédition de l'envoi est **acceptée**

☐ l'expédition de l'envoi est **refusée** au motif suivant :

Le Vétérinaire-Inspecteur
Dr S. PELLARIN

(date, sceau et signature de l'autorité compétente)

RÉPUBLIQUE FRANÇAISE
DDCSPP 87

Epidémiologie du Toxoplasma gondii en Thaïlande

Toxoplasma gondii est un parasite intracellulaire obligatoire. L'infection par *T. gondii* est largement répandue dans le monde entier. Néanmoins, elle est peu étudiée dans les pays d'Asie du Sud-est dont la Thaïlande.

Nous avons réalisé 3 travaux sur le terrain en Thaïlande pour essayer de comprendre la circulation de ce parasite à travers une étude de séroprévalence chez des poulets en zone rurale et des essais d'isolement de souches chez les animaux en vue d'un génotypage. Lors des deux premières missions de terrain dans deux villages de la province de Kanchanaburi, nous avons cherché à déterminer la séroprevalence de l'infection chez des poulets *(Gallus domesticus)* en utilisant 2 tests sérologiques, Modified-Agglutination Test (MAT et immunofluorescence indirecte (IFAT) puis à isoler des souches de *T. gondii* à partir des animaux séropositifs. Lors de la troisième mission réalisée dans 3 autres provinces thaïlandaises (Nakhonratchasima, Lopburi et Saraburi), nous avons essayé d'isoler directement le parasite à partir de carcasses de poulets vendues sur les marchés ou d'autres animaux trouvés morts.

La séroprévalence globale pour les 2 premières misions sur 600 poulets du Kanchanaburi était de 17,7% (IC 95% : 14,6-20,7) et 33,0% (IC 95% : 29,2-36,8), par MAT et IFAT respectivement. Le calcul du coefficient κ montre une absence de concordance entre les deux tests.

Au total, 162 essais d'isolement ont été effectués par inoculation à des souris, mais aucune souche viable de *T. gondii* n'a été isolée pendant ces 3 travaux sur le terrain. Cependant, nous avons détecté la présence d'ADN toxoplasmique en qPCR ciblant le gène 529 bp dans 13 culots de digestion d'organes de poulets, pigeon, caille et dans des cerveaux ou cœurs de souris inoculés par 16 autres poulets. Les Ct observés en qPCR étaient ≥33 indiquant une faible quantité d'ADN parasitaire dans nos échantillons qui n'a pas permis une caractérisation génétique par marqueurs microsatellites.

Ce travail a démontré l'importance et les difficultés du travail de terrain pour l'étude de séroprévalence ainsi que l'étude d'isolement. L'isolement des souches de *T. gondii* a demandé un travail d'échantillonnage intensif, complexe dans l'environnement tropical et humide de la Thaïlande. Les différents paramètres ayant pu avoir un impact négatif sur nos résultats sont discutés. Ils expliquent l'absence d'isolement de souches chez des animaux séropositifs.

Mots-clés : *Toxoplasma gondii*, Thaïlande, séroprévalence, génotype, environnement tropical

Epidemiology of Toxoplasma gondii in Thailand

Toxoplasma gondii is an obligate intracellular parasite. *Toxoplasma gondii* infection is widespread throughout the world. Nevertheless, it is poorly studied in Southeast Asian countries including Thailand.

We carried out 3 field works in Thailand to try to understand the circulation of *T. gondii* through a seroprevalence study in chickens in rural areas and strain isolation attempts in animals. During the two first field works, performed in Kanchanaburi province, we determined the seroprevalence in chickens (*Gallus domesticus*) using 2 serological tests, a Modified-Agglutination-Test (MAT) and an immunofluorescence assay (IFAT) and subsequently tried to isolate the strains of *T. gondii* from seropositive animals. During the third field work carried out in 3 other Thai provinces (Nakhonratchasima, Lopburi and Saraburi), we attempted to isolate strains directly from chicken carcasses sold in different markets or other dead animals.

The overall seroprevalence for 600 chickens sampled over the two field works in Kanchanaburi was 17.7% (95% CI: 14.6% -20.7) and 33.0% (95% CI: 29.2-36.8), by MAT, and IFAT, respectively. The κ coefficient indicated an absence of concordance between the 2 serological tests.

A total of 162 isolation attempts were performed by mouse bioassays, but no viable strain of *T. gondii* was isolated during these 3 field works. However, a qPCR targeting 529 bp *T. gondii* gene was positive for 13 digestion pellets of organs of chickens, pigeon, quail and in brains or hearts of mice inoculated with 16 other chickens. These qPCR were weaklly positive (Ct ≥33) indicating a low amount of parasite DNA in our samples that did not allow genotyping *T. gondii* with microsatellite markers.

This work demonstrated the importance and difficulties of field work for the seroprevalence study as well as strain isolation. The isolation of *T. gondii* strains required intensive and complex sampling in the tropical and humid environment of Thailand. The diverse factors that could have a negative impact on our results are discussed. They might explain the absence of strain isolation from seropositive animals.

Keywords: *Toxoplasma gondii*, Thailand, seroprevalence, genotype, tropical environment