รายงานวิจัยฉบับสมบูรณ์

การศึกษาชนิดสายพันธุ์ของเชื้อ *Toxoplasma gondii* ในประเทศไทย

Determination of *Toxoplasma gondii* typing strains in Thailand

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CONTENTS

LIST OF TABLES

Tables **Page**

LIST OF FIGURES

ACKNOWLEDGEMENTS

ABSTRACT

Toxoplasma gondii is an intracellular parasite that causes the disease called toxoplasmosis in humans and animals worldwide. This study was aimed to investigate the seroprevalence of *Toxoplasma* infection and molecular genotyping of *T. gondii* isolated from humans, ratail meat samples and cat feces. Serum from 1,400 pregnant women and 735 HIV-infected patients were determined for anti-*Toxoplasma* antibodies using ELISA method. The overall seroprevalence of *Toxoplasma* infection among pregnant women was 30.9% (432/1,400). The seropositive rate of only anti-*Toxoplasma* IgG antibodies or only IgM seropositivity was 21.8% (305/1,400) and 4.4% (61/1,400) respectively. 4.6% (66/1,400) of pregnant women were tested positive for both IgG and IgM. In HIV-infected patients, the overall seroprevalence of *Toxoplasma* infection was 27.5% (202/735). Out of the 735 HIV subjects, 26.4% (194/735) and 0.8% (6/735) were positive for only *Toxoplasma* IgG or only IgM respectively. Two cases were seropositive for both IgG and IgM. The proportion of *Toxoplasma* seroprevalence among pregnant women and HIV cases found in this study indicated the highly risk of contracting *Toxoplasma* infections; thus, the knowledge about preventive measures of this infection should be continuously informed in these people to decrease the important risk of congenital toxoplasmosis during pregnancy and to minimize the risk of reactivation of latent infection among HIV-infected patients. The DNA was extracted from blood samples of all HIV cases and 432 seropositive pregnant women to confirm the presence of *T. gondii* DNA using PCR amplification of the 529 bp repeat element. The results from all tested samples were negative for *T. gondii* DNA. Two hundreds of pork and beef meat obtained from 5 different part of each animal including brain, heart, liver, lung, and tenderloin were also negative for *T. gondii* tissue cysts when detected by PCR. This finding indicated that *Toxoplasma* infections in humans in this studied area may be not obtained from eating raw meat. We were then analyzed the molecular genotyping of 13 *T. gondii* isolated from some cat feces obtained from Hat Yai area. The PCR-RFLP patterns of *SAG1, SAG2*-new, *SAG3, BTUB* and *GRA6* markers were revealed that 1 (7.7%) isolate was type I, 2 (15.4%) isolates were type III and 1 (7.7%) isolate was type II or type III. In addition, 4 *T. gondii* isolates were characterized as mixed genotypes of alleles at different loci. The remaining 5 *Toxoplasma* isolates were not able to amplify with all markers. The presence of unusual genotypes indicated that these isolates may possibly have new virulent traits that are able to cause *Toxoplasma* infections more

severe. This is the first report of genotypic characteristics of *T. gondii* isolated from naturally infected animals in Thailand.

บทคัดย่อ

Toxoplasma gondii เป็นเชื้อปรสิตที่อาศัยอยู่ในเซลล์ ซึ่งทำให้เกิดโรคท็อกโซพลาสโมซิสทั้งในคน และสัตว์ทั่วโลก การศึกษานี้มีวัตถุประสงค์เพื่อตรวจหาความชุกของแอนติบอดีต่อการติดเชื้อ *T. gondii* และ ศึกษาชนิดสายพันธุ์ของเชื้อที่แยกได้จากคน เนื้อสัตว์ และอุจจาระแมว โดยเก็บตัวอย่างซีรัมจากหญิงมีครรภ์ จำนวน 1,400 ราย และผู้ติดเชื้อเอชไอวีจำนวน 735 ราย นำมาตรวจหาแอนติบอดีต่อ *T. gondii* ด้วยวิธี ELISA ผลการทดลองพบว่า หญิงมีครรภ์มีความชุกของการติดเชื้อรวมเท่ากับ 30.9% (432/1,400) โดย 21.8% (305/1,400) และ 4.4% (61/1,400) ให้ผลบวกต่อแอนติบอดีชนิด IgG หรือ IgM เพียงอย่างเดียว ตามลำดับ และ 4.6% (66/1,400) ให้ผลบวกต่อแอนติบอดีทั้งชนิด IgG และ IgM ส่วนผู้ติดเชื้อเอชไอวี พบ ความชุกของการติดเชื้อรวมเท่ากับ 27.5% (202/735) โดย 26.4% (194/735) และ 0.8% (6/735) ให้ ผลบวกต่อแอนติบอดีชนิด IgG หรือ IgM เพียงอย่างเดียวตามลำดับ และมี 2 รายที่ให้ผลบวกต่อแอนติบอดี ทั้งชนิด IgG และ IgM จากผลการทดลองบ่งชี้ว่าหญิงมีครรภ์และผู้ติดเชื้อเอชไอวีมีความเสี่ยงสูงต่อการติดเชื้อ *T. gondii* ดังนั้น ควรมี การให้ความรู้เกี่ยวกับวิธีการป้องกันการติดเชื้อแก่ผู้ป่วยเหล่านี้อย่างต่อเนื่องเพื่อช่วย ลดความเสี่ยงต่อการติดเชื้อจากมารดาไปยังทารกในครรภ์ และลดความเสี่ยงของการกลับเป็นซ้ำของการติด เชื้อแบบแอบแฝงในผู้ติดเชื้อเอชไอวี การตรวจหาดีเอ็นเอของ *T. gondii* จากตัวอย่างเลือดทุกรายของผู้ติด เชื้อเอชไอวีและหญิงมีครรภ์ที่ให้ผลบวกต่อการตรวจหาแอนติบอดีต่อ *T. gondii* จำนวน 432 ราย ด้วย วิธีการเพิ่มปริมาณของชิ้นส่วน 529 bp repeat element โดยวิธี PCR พบว่า ตัวอย่างที่ทดสอบตรวจไม่พบ ดีเอ็นเอของ *T. gondii* นอกจากนี้ การตรวจหา tissue cysts ของ *T. gondii* จากชิ้นส่วนที่แตกต่างกันของ ตัวอย่างเนื้อสุกรและโค ได้แก่ สมอง หัวใจ ตับ ปอด และเนื้อสะโพก จำนวน 200 ตัวอย่าง โดยวิธี PCR พบว่า ตัวอย่างที่ทดสอบ ทั้งหมดตรวจไม่พบ tissue cyst ของเชื้อ *T. gondii* ผลการทดลองนี้แสดงให้เห็นว่า การติดเชื้อของคนที่อยู่ในบริเวณที่ทำการศึกษานี้อาจไม่ได้ติดเชื้อมาจากการรับประทานเนื้อสัตว์ดิบ ต่อมา ผู้วิจัยได้ศึกษาลักษณะสายพันธุ์ของเชื้อ *T. gondii* ด้วยวิธี PCR-RFLP โดยวิเคราะห์รูปแบบดีเอ็นเอของยีน *SAG1, SAG2*-new, *SAG3, BTUB* และ *GRA6* จากเชื้อจำนวน 13 สายพันธุ์ที่แยกได้จากตัวอย่างอุจจาระ แมวภายในเขตพื้นที่ของอำเภอหาดใหญ่ ผลการทดลองพบเชื้อ *T. gondii* สายพันธุ์ชนิด type I จำนวน 1 สายพันธุ์ (7.7%) สายพันธุ์ชนิด type III จำนวน 2 สายพันธุ์ (15.4%) และสายพันธุ์ชนิด type II หรือ type III จำนวน 1 ตัวอย่าง (7.7%) นอกจากนี้ พบเชื้อ *T. gondii* จำนวน 4 สายพันธุ์ที่เป็นสายพันธุ์ผสม (mixed

genotypes) ซึ่งมีการผสมของอัลลีลในตำแหน่งของยีนที่ทดสอบ ส่วนเชื้อ *T. gondii* อีก 5 สายพันธุ์พบว่า ไม่สามารถเพิ่มจำนวนยีนที่ทำการศึกษาได้ทุกยีน การตรวจพบเชื้อ *T. gondii* ชนิดสายพันธุ์ที่มีความแตกต่าง จากสายพันธุ์ดั้งเดิมแสดงให้เห็นว่า เชื้อสายพันธุ์เหล่านี้อาจมีความเป็นไปได้ที่จะมียีนก่อโรคชนิดใหม่   ที่ สามารถทำให้เกิดการติดเชื้อ *T. gondii* ที่มีความรุนแรงมากขึ้น

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite that causes toxoplasmosis in humans and a variety of economically important animals. It is estimated that 30% to 40% of the world's population are seropositive for *Toxoplasma* infection depending on geographical location (Tenter *et al.,* 2000). The major mode of transmission of *T. gondii* among humans are acquired by eating raw or undercooked meat containing viable tissue cysts or by accidentally ingesting food or water contaminated with sporulated oocysts shed by cats and other felines into the environment (Dubey, 2004; Clementino *et al*., 2007). Furthermore, this disease can be also transmitted from infected pregnant women to their fetus by transplacental transmission which, in this case, may cause mental retardation, blindness, epilepsy, and death (Hill and Dubey, 2002).

Seroprevalence in *Toxoplasma* infection varies globally depending on individual subpopulations (Pappas *et al*., 2009; Flatt and Shetty, 2012), religions (Al-Harthi *et al*., 2006; Chan *et al*., 2009), socioeconomic practices (Alvarado-Esquivel *et al*., 2012) and methods used for detection (Chemoh *et al*., 2013). The example of seroprevalences for *Toxoplasma* infection includes 59% in Brazil (Ferezin *et al*., 2013), 3.98% in China (Hua *et al*., 2013), 83.6% in Ethiopia (Zemene *et al*., 2012), 75% in India (Chintapalli and Padmaja, 2013), 10.3% in Japan (Sakikawa *et al*., 2012), 28.3% in Thailand (Nissapatorn *et al*., 2011) and 49% in Malaysia (Nissapatorn *et al*., 2003). In Thailand, serological studies against *Toxoplasma* infection in HIV infected patients was reported as high as 22.4% to 43.2% (Sukthana *et al*., 2000; Nissapatorn *et al*., 2001), but in pregnant women and in newborns, the infection rate were varied from 2.3% to 29.6%, and 7.18% to 13.14% respectively (Nabnien, 1979; Morakote *et al*., 1984; Chintana *et al.,* 1998; Wanachiwanawin *et al*., 2001).

Toxoplasma infection in healthy adult is usually asymptomatic or mild condition, however, it can cause severe complication in immunocompromised patients especially those with acquired immunodeficiency syndrome, with immunosuppressive cancer, and transplant recipients who are on immunosuppressive drugs (Hill and Dubey, 2002; Montoya and Liesenfeld, 2004; Hill *et al.*, 2005). The development and severity of the disease are different in individual due to major factors including hosts and parasite genetics (Montoya and Liesenfeld, 2004). Other factors which are likely to influence the virulence including the completion of the immunological systems and age of the host (Holland, 2009), while the severity of infection in animals depends on the *T. gondii* strain differently (Sibley and Boothroyd, 1992). Signs and symptoms of toxoplasmosis involving the eyes and/or the

central nervous system were highly reported as 43.2% in HIV positive patients (Sukthana *et al*., 2000).

Many studies reported *Toxoplasma* infection in animals for human consumption (Bangoura *et al*., 2013; Hill and Dubey, 2013; Kang *et al*., 2013; Lopes *et al*., 2013) and having a close contact with cats and cleaning their litter may transmit the disease to pregnant women (Fakhfatk *et al*., 2013). Other factors associated with *Toxoplasma* infection among pregnant women from many studies revealed that causes of *Toxoplasma* infection were also due to contact with cats, the consumption of raw or undercooked meat, the residence and woman's age (Elbez-Rubinstein *et al*., 2009; Sakikawa *et al*., 2012; Mwambe *et al*., 2013).

Apart from the host immune status, the genotype of *Toxoplasma* has been extensively studies whether its characteristics may influence the course of disease. *T. gondii* isolates from humans and animals in North America and Europe were classified into 3 dominant lineages (type I, II and III) which each type caused different virulence in mice (Sibley and Boothroyd, 1992; Howe and Sibley, 1995). However, recent genotyping studies in South America have found *Toxoplasma* hybrid isolates or atypical strains which were more virulent and highly diverse than in North America and Europe (Darde, 2004; Khan *et al.*, 2006; Lehmann *et al*., 2006). The evidence of toxoplasmosis in humans has been reported that patients were mainly infected by *Toxoplasma* type I or II strains, whereas animals were infected with most type III strains (Howe and Sibley, 1995; Ajzenberg *et al*., 2002a). To the best of our knowledge, there is none on genotyping of *T. gondii* strains both in humans and animals has been reported from Thailand.

Therefore, this study aims to determine the molecular genotyping of *T. gondii* isolated from humans, meat samples and cat feces which were proved to associate with *Toxoplasma* infection. The finding will provide the better understanding of the epidemiology of *Toxoplasma* infection and the source of the parasite contamination which are necessary for investigating the cause of disease transmission and to evaluate the potential virulence of these isolates in humans and animals.

OBJECTIVES

1. To determine the seroprevalence of antibodies against *Toxoplasma* infection in pregnant women and HIV patients by ELISA test.

2. To examine *T. gondii* DNA in the patients by Polymerase Chain Reaction (PCR) method.

3. To evaluate the prevalence of *T. gondii* tissue cysts in meat samples from retail meat stores using PCR analysis.

4. To determine the genotyping of *T. gondii* strains using PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) technique.

LITERATURE REVIEW

1. *Toxoplasma gondii*

1.1 Taxonomy

    T. gondii is an obligate intracellular protozoan pathogen which belongs to the phylum Apicomplexa, the family of the *Toxoplasmatidae* and the subclass of the Coccidiasina. The dominant characteristic of *Apicomplexa* is the presence of apical end that has a structurally polarized cell and a complex arrangement of their cytoskeletons and organelles which plays a role to attack the host cell (Frenkel *et al.,* 1970; Dubey *et al.*, 1998). *T. gondii* was first discovered by Nicolle and Manceaux isolated from the North African rodent (*Ctenodactylus gondi*) (Nicolle and Manceaux, 1908). The *Toxoplasma* genus has only one species and its name derived from the combination of two words in Greek (*toxon* and *plasma*) which refers to this organism having a crescentshape like structure.

1.2 Parasite stages

    T. gondii has three infectious stages, including the tachyzoites, the bradyzoites, and the sporozoites. The parasite in all infective stages is similar crescent-shaped cells with a pointed apical end and a rounded posterior end.

    The tachyzoite has an approximate length 5 μm and width 2 μm (Smith, 1995). It is identified as a rapidly diving form which found during an acute phase or recurrence of a chronic infection. When tachyzoites penetrate host cells, they can proliferate within a membrane-enclosed structure called the parasitophorous vacuole (PV). Although the mechanism of tachyzoite's movement is not clear, they can move through the gliding, flexing and rotating (Dubey *et al*., 1998). The detection of the tachyzoite form in patients is an indicator of symptoms caused by toxoplasmosis and this form is sensitive to the drugs for treatment.

    Bradyzoites is converted from tachyzoite which is occurred by stress that arises from the development of the immune system during this infection. They are a slowly replicating stage found in the tissues of the host cells in the cyst form. Because of the thickness of the bradyzoite cyst wall is less than 0.5 μm, making it is flexible and easy to expand. These cysts contain hundreds or thousands of bradyzoites. The size of tissue cysts varies from 10 μm to 100 μm and depends on their age. The cysts in different tissues are found to have a different shape. Another important feature of tissue cysts is resistant to degradation by proteolytic enzymes secreted from the host stomach, thereby enabling it to be transmitted by oral ingestion (Jacobs *et al*., 1960). Bradyzoite cysts are found predominantly in the brain, eye,

skeletal muscle and cardiac tissue. However, they may reside in visceral organs, including the lungs, liver and kidneys. Because of a low immunogenicity of bradyzoites and their capability partially in protection the host from parasite-induced cell rupture and immunopathology, these cysts can persist as viable parasites for the lifespan of the host without causing an inflammatory response to the host (Bohne *et al*., 1999; Lyons *et al*., 2002; Kim and Boothroyd, 2005).

The sporozoite form can be found in sporulated oocysts. Its structure is similar to tachyzoites, but it is different in the high number of micronemes, rhoptries and amylopectin granules than tachyzoites (Dubey *et al*., 1998). Within 21 days of excretion, sporulation takes place outside the cat's body which in accordance with temperature and aeration that it exposes (Dubey *et al*., 1970). There are eight infective sporozoites resided in each sporulated oocyst. The size of the sporulated oocyst is about 12x13 μm and their shape is oval. The oocyst wall is composed of a double layer which the inner layer is thicker than the outer layer (Speer *et al*., 1998). The main component found in the outer oocyst layer only (Possenti *et al.,* 2010) or in the entire wall fractions (Mai *et al*., 2009; Fritz *et al*., 2012) is cystein and tyrosine rich proteins resulting in the oocyst wall is structurally robust. The oocyst wall can prevent parasites from a variety of physical and chemical factors, including disinfectants such as UV, ozone, and chlorine-based products (Dumètre and Dardé, 2003; Belli *et al*., 2006; Jones and Dubey, 2010). Moreover, the oocysts can survive for months and even years in moist environmental conditions (Mai *et al*., 2009).

1.3 Mode of transmission

There are four major routes that humans can contract with infectious of *T. gondii* (figure 1). Firstly, people can be acquired by consumption of food or water contaminated with oocysts excreted in the feces of infected cats. Secondly, the ingestion of raw or undercooked meat containing the bradyzoite form in tissue cysts may cause *Toxoplasma* infection (Dubey, 1998a). Thirdly, pregnant women who infected with primary infection during gestation can transmit the tachyzoite to her developing fetuses by congenital transmission. And lastly, this route is rarely found by acquiring the tachyzoite or bradyzoite from organ transplantation or blood transfusion (Montoya and Liesenfeld, 2004). The cause of *Toxoplasma* infection in humans mainly results from an accidental ingestion of the parasite infective stages. It has never been reported the infection caused by transmission from person to person directly or breastfeeding. The infection in definitive hosts can occur by eating oocysts contaminated from environmental or cysts of infected preys.

1.4 Strains of *T. gondii*

The genotyping of *T. gondii* in North America and Europe based on multi-locus enzyme electrophoresis, PCR–RFLP and microsatellite typing have divided the parasite isolated from both humans and animals into 3 main types (Types I, II and III) (Howe and Sibley, 1995; Ajzenberg *et al*., 2002b; Boothroyd and Grigg, 2002). Although three *T. gondii* groups have 98% similarity at DNA level, the virulent characteristic presents in mouse model are clearly different. Type I strains are the most virulent causing mice die rapidly when infection with <10 tachyzoites (lethal dose (LD_{100}) = 1), by contrast, intermediate virulent strains (type II) and low virulent strains (type III) allow survival after inoculation of more than > 10^3 tachyzoites (LD_{100} > 10^3) (Sibley and Boothroyd, 1992). The epidemiological pattern of occurrence in 3 predominating strains is differently found (Montoya and Liesenfeld, 2004). Type II strains are most predominantly associated with human toxoplasmosis, both in AIDS patients and congenital disease. Type I strains have been found in patients with congenital disease, whereas type III strains are mostly isolated from animals (Howe and Sibley, 1995; Ajzenberg *et al*., 2002b).

Recent genotypic studies of *T. gondii* strains isolated from South America, Africa, and Asia show a high diversity of their genotypes isolated from humans (Khan *et al.,* 2006; Ferreira *et al.,* 2008) and animals indicating a history of more frequent genetic exchanges and genetic drift (Ajzenberg *et al*., 2004; Lehmann *et al*., 2004). These isolates are clustered as new haplogroups which they are different from clonal lineages previously reported (with the exception of type III, which is really cosmopolitan) (Khan *et al.,* 2007; Pena *et al.,* 2008; Mercier *et al.,* 2010). Until now, 12 haplogroups (including the 3 initially described lineages, types I, II, and III) have been explained based on sequence-based analyses (Khan *et al.,* 2007; Khan *et al.,* 2011). However, these haplogroups still remain truly atypical and highly diverse isolates with many unique polymorphisms which cannot be clustered into one of these haplogroups (Mercier *et al.,* 2011). This may be due to geographical origins and phenotypic characteristics. In South America, additional clonal lineages isolated from animals which are commonly found and endemic in Brazil are type Br I to IV haplogroups (Ajzenberg *et al*., 2004; Pena *et al*., 2008), whereas the detection of type II isolates is rarely reported (Dubey *et al*., 2006). In Africa, atypical strains known as the Africa 1 to 3 haplogroups were found together with type II and III lineages (Velmurugan *et al.,* 2008; Ajzenberg *et al*., 2009; Mercier *et al*., 2010). In Asia such as China, Sri Lanka, and Vietnam, a genetic diversity of *Toxoplasma* isolates is lower than in South America (Dubey *et al*., 2007a; Dubey *et al*., 2007c; Zhou *et al.,* 2010). The virulent phenotypes of these atypical

strains or other clonal lineages ranged from the non-virulent to moderate or highly virulent phenotype according to differences in the combination of genes that they have inherited (Grigg and Suzuki, 2003; Saeij *et al.,* 2005; Darde, 2008). Interestingly, a high predominance of atypical strains and another clonal haplogroup (haplogroup 12) close to type II (Khan *et al*., 2011) is recently found in North America from various wild animals as well as domestic animals (Dubey *et al*., 2008a; Dubey *et al*., 2008b).
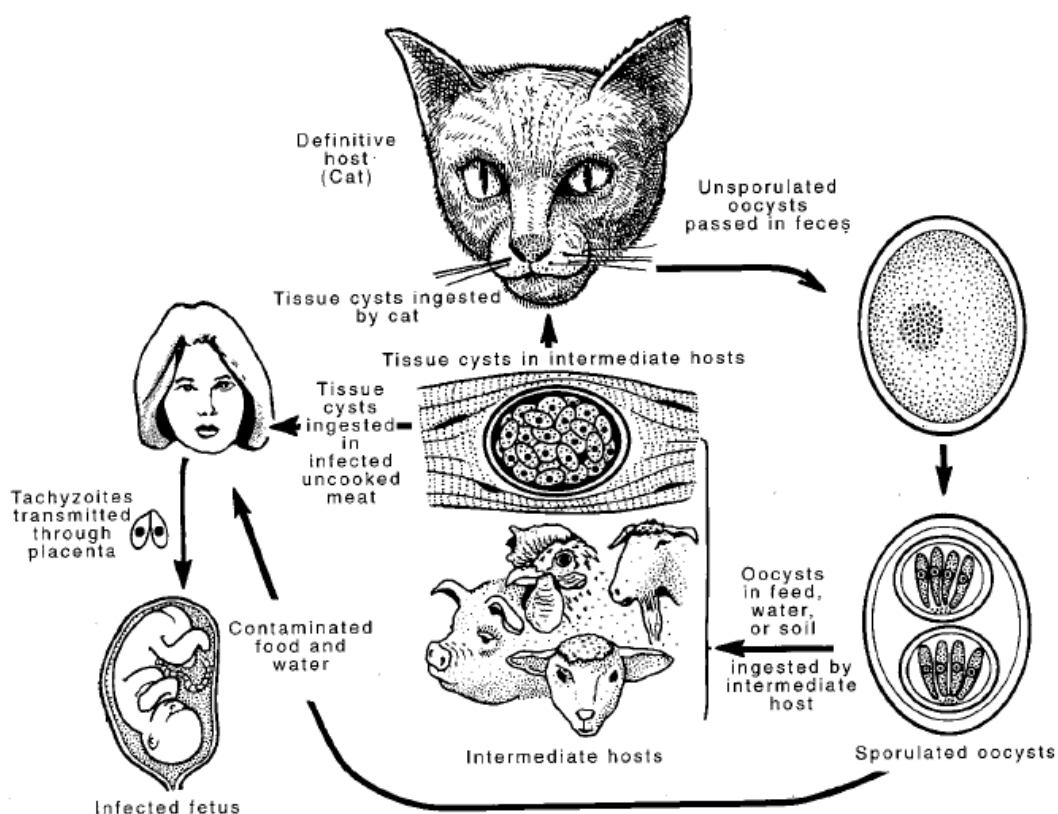
Figure 1 Pathways of *T. gondii* infection (Dubey *et al.,* 1998)

2. Epidemiology of *Toxoplasma* infection in humans and animals

The prevalence of *Toxoplasma* infection is worldwide in distribution. Approximately, one third of the world human population has been exposed to the parasite (Pappas *et al.*, 2009). However, the seroprevalence in humans varies greatly between different continents and countries (Tenter *et al.*, 2000; Sukthana, 2006). In Latin America and tropical African countries, *Toxoplasma* infection is highly prevalent. In Central and Southern Europe, the prevalence have been observed moderately, whereas in North America, South East Asia, Northern Europe and Sahelian countries of Africa, it has been found low seroprevalence (10% to 30%). In addition, the prevalence also differs among different geographical areas

within the same country. For instance, the seroprevalence in the USA varies from 29.2%, 20.5% and 17.5% in the northeast, the south-mid west and the west respectively (Jones *et al*., 2001). In regions where the environment are not favor for the oocyst survival such as colder or drier areas and high altitudes, *Toxoplasma* infection occurs at low prevalent rates, contrarily, high prevalent rate is seen in tropical countries with a humid and warm climate. Many studies have been reported other factors that involved in the different seroprevalence of *Toxoplasma* infection, including individual subpopulations (Pappas *et al*., 2009; Flatt and Shetty, 2012), religions (Al-Harthi *et al*., 2006; Chan *et al*., 2009) and socioeconomic level (Alvarado-Esquivel *et al*., 2012). However, these obtained seroprevalence data from different serological methods show varying in the sensitivity and specificity.

T. *gondii* can cause infections in many kinds of meat-producing animals. The locations and number of its tissue cysts resided in these animals are different in animal species (Dubey *et al*., 1998). It has been well known that a major route of transmission to humans is the ingestion of raw or undercooked meat especially pigs and sheep. However, recent studies from European countries reported that *Toxoplasma* seroprevalence in fattening pigs has a significant decrease (< 10%) because they use the intensive farm management with sufficient standards of hygiene and confinement (Tenter *et al*., 2000). The current seroprevalence in slaughter pigs occurs less than 5% in most industrialized countries in the United State (Dubey and Jones, 2008). The changes in animal farms using hygienic measures are possible to reduce the risk of *Toxoplasma* infection in livestock. When comparison to sheep and goats, their farm systems has still not changed and continued to be outdoor production systems. The seroprevalence of *T. gondii* in free-ranging sheep and goats in many region of the world revealed to be 92% and 75% respectively, which is resulted from keeping the animals on pastures leading to an increase of the chance of them to the exposure with contaminated oocysts from the environment (Tenter *et al*., 2000). The seroprevalence in other animals such as poultry and horses are lower and more different (Dubey, 2010). In cattle, this infection remains unclearly in conclusions. Some studies reported the seropositivity rates in cattle vary from 2 to 92% (Tenter *et al*., 2000), whereas another study has not detected *Toxoplasma* infection in retail beef meat (Dubey *et al*., 2005). However, other studies have been indicated that cattle may be resistant to *Toxoplasma* infection, and it is possible to clear the infection (Dubey and Thulliez, 1993; Opsteegh *et al*., 2011).

The felines are considered as the only species of a definitive host for *T. gondii* and therefore they have a major role in the epidemiology of the parasite infection. The infected

cats are found commonly in both domestic cats and wild felines. The prevalence of *Toxoplasma* infection in adult domestic cats depends on the type of feed used and cats' lifestyles (indoor or outdoor) which may be found as high as 74% of their populations (Tenter *et al*., 2000). Additionally, it is also found that the environment or the place where cats lived is the one factor which can influence the prevalence of infection. Stray or feral cats have higher seroprevalence rates than cats living in an urban or suburban area. In Asia, the seroprevalence of latent *Toxoplasma* infection in pet cats is lower (range from 6% to 9%) than in Europe, South America, and the USA (range from 9% to 46%) (Tenter *et al*., 2000).

In Thailand, the information of *Toxoplasma* infection in humans and animals has been reported with greatly variation. The seroprevalence in pregnant women were range from 2.5% to 28.3% (Morakote *et al*., 1984; Maleewong *et al*., 1989; Chintana, 1991; Chintana *et al*., 1998; Sukthana, 1999; Tantivanich *et al*., 2001; Nissapatorn *et al*., 2011) whereas in HIV patients were range from 22.4% to 53.7% (Wanachiwanawin *et al*., 2001). In animals, the prevalence of *Toxoplasma* infection reported was 9.4% in dairy cows in the North (Inpankaew *et al*., 2010) and 22.3% in the Northeast (Jittapalapong *et al*., 2008), 13.0 % in elephants (Wiengcharoen *et al.*, 2012), 27.9% in goats (Jittapalapong *et al*., 2005), and 4.8% in stray cats (Jittapalapong *et al*., 2010).

3. Clinical symptoms

Infection with *T. gondii* in humans can cause toxoplasmosis which presents with varied signs and symptoms, according to host factors such as genetic background and immune status, as well as parasite characteristics such as virulence of the strain and inoculum size (Montoya and Liesenfeld, 2004).

3.1 Immunocompetent subjects

Primary acquired infection with *T. gondii* is usually asymptomatic in most cases of immunocompetent adults and children (Montoya and Liesenfeld, 2004). Only 10 to 20% of patients develop lymphadenitis or mild illness characterized by fever, malaise, myalgia, headache, sore throat and lymphadenopathy. The disease may persist for several weeks in some cases similar to infectious mononucleosis. The symptoms are a self-limited and non-specific illness without treatment. A recently acquired *Toxoplasma* infection may rarely cause acute toxoplasmic chorioretinitis with visual impairment (Delair *et al.,* 2008), although it was previously thought that ocular toxoplasmosis was the late outcome of congenital infection.

3.2 Immunocompromised patients

The outcome of *Toxoplasma* infection in immunocompromised patients (AIDS patients, organ transplant recipients, and cancer patients) contrasts greatly to adults with normal immune function. Toxoplasmosis is a major public health problem in this clinical group, especially in AIDS patients, because it is often severe diseases including splenomegaly, chorioretinitis, pneumonitis, encephalitis, multisystem organ failure, and even death (Montoya and Liesenfeld, 2004). It has been reported that host factors are more important than parasite factors in patient resistance and susceptibility to *Toxoplasma* infection in the immunocompromised patients (Ajzenberg *et al.,* 2009). The reactivation of latent infection through cyst rupture is an important cause of recurrent infections in these individuals (Porter and Sande, 1992), but their risk is different based on the group of patients. In patients infected with HIV, they have a high risk to develop acute *Toxoplasma* infection if their CD4+ T-lymphocyte count drops less than 100 cells/µL or if below 200 cells/µL when the opportunistic infection or malignancy occurs simultaneously (Martinez *et al.*, 2002; Jayawardena *et al.*, 2008). Toxoplasmosis encephalitis (TE) is one of the most frequent opportunistic infections, and is the most common cause of focal brain lesions among persons with AIDS, typically in the later stages of HIV infection (Luft and Chua, 2000; Nissapatorn *et al.*, 2004; Valenta *et al.*, 2009). Although, the central nervous system (CNS) is the main target site that is affected by this infection, the disease can occur in eyes and lungs which are the most frequent sites of extracerebral toxoplasmosis (ECT). Cerebral toxoplasmosis (CT) presents more frequently than ECT (Rabaud *et al.,* 1994). Clinical manifestations of TE vary from a subacute gradual process evolving over weeks to an acute confusional state, with or without focal neurological deficits, evolving over days. Moreover, they also present as mental status changes, seizures, focal motor deficits, cranial nerve disturbances, sensory abnormalities, cerebellar signs, neuropsychiatric findings, and movement disorders. Hemiparesis and speech abnormalities are the most common focal neurological findings (Luft *et al.*, 1993). Retinochoroiditis, pneumonia or other disseminated systemic disease can be observed in HIV-infected subjects but are less common than TE (Liesenfeld *et al.*, 1999). However, the recent widespread use of highly active antiretroviral therapy (HAART) has declined the incidence of AIDS-associated toxoplasmic encephalitis (TE) in developed countries (Sacktor *et al.*, 2001; d' Arminio Monforte *et al.*, 2004). In organ transplant patients, clinically severe toxoplasmosis can occur from receiving a cyst-containing organ from an infected donor given to an uninfected recipient (Botterel *et al.*,

2002; Rogers *et al*., 2008; Martina *et al*., 2011) that heart transplants were the most at risk (Gallino *et al*., 1996; Sanchez Mejia *et al*., 2011).

3.3 Congenital toxoplasmosis

Most pregnant women acquired acute *Toxoplasma* infection had no apparent symptoms (Boyer *et al*., 2005; Remington *et al*., 2006). Unusually, pregnant women will present visual alterations caused by toxoplasmic chorioretinitis (Garweg *et al*., 2005) due to primary acquired infection or recurrence of chronic infections. Newly acquired *Toxoplasma* infection in pregnant women during gestation can be transmitted to unborn fetus via placenta and consequently causes congenital toxoplasmosis (Remington *et al*., 2006). The risk of mother-to-child transmission and the severity of congenital damage vary depending on the gestational age at maternal infection (Dunn *et al*., 1999). Once maternal infection during the first trimester, the risk of congenital infection decreases (10-15%), whereas it highly increases (60-90%) in the third trimester (Dunn *et al*., 1999). On the contrary, the severity of disease is the highest in the first trimester and lowest in the third trimester (Holliman, 1995; Remington *et al*., 2006). Although, most neonates are asymptomatic when their infections occur within the third trimester (Desmonts and Couvreur, 1974), these infants may later develop ocular toxoplasmosis or neurological symptoms in the second or third decade of life (Roberts and McLeod, 1999; Boyer, 2000; Jones *et al*., 2003). The effects of transplacental transmission during the first trimester to fetal development are often severe abnormalities resulting to spontaneous abortion or still-birth. Necrosis foci and strong inflammation caused by an induction of parasite replication leads to irregularities in the brain and eye tissue. Significant manifestations in the fetus also include mental retardation, seizures, microcephalus, hydrocephalus, intracranial calcifications, chorioretinitis, deafness, psychomotor deficiency and blindness (McAuley *et al*., 1994; Remington *et al*., 2006). The classical triad of congenital toxoplasmosis such as hydrocephalus, microcephalus, cerebral calcifications and chorioretinitis is rarely found in a live infant.

4. Diagnosis

As a result of clinical symptoms of *Toxoplasma* infection are nonspecific, the diagnosis is therefore unreliable from the clinical symptoms point of view. This infection can be diagnosed usually by many methods divided into two major ways including indirect methods (serological tests) and direct methods (bioassays in mice and/ or cats, and molecular methods) (Hill and Dubey, 2002; Montoya, 2002; Lappalainen and Hedman, 2004; Remington *et al*., 2004).

4.1 Indirect methods: serological methods

Serological methods are used for detection of specific anti-*T. gondii* antibodies in human and animals (Dubey and Beattie, 1988; Remingtion *et al*., 2006). In human, serological detection should be done in pregnant women and immunocompromised patients (Montoya and Liesenfeld, 2004). Serological detection is the initial and primary method of diagnosis of *Toxoplasma* infection. These serological techniques differ in their sensitivity and specificity. Currently, different serological tests are commonly used including the Sabin-Feldman dye test (DT), Indirect fluorescent antibody test (IFA), Indirect hemagglutination test (IHA), Enzyme-linked immunoabsorbent assay (ELISA), modified direct agglutination test (MAT). Another diagnostic serology that has a different purpose from these tests is avidity test which can differentiate between recently acquired infections and distantly acquired infections.

Sabin-Feldman dye test (DT)

The DT was the first system which can detect *Toxoplasma* specific antibody at low levels (Sabin and Feldman, 1948). It is still considered as the gold standard serological test for *T. gondii* antibodies detection in humans. The DT measures complement fixing antibodies. In this test, the patient serum is incubated with live tachyzoites and a complement-like accessory factor at 37°C for 1 h. After incubation, methylene blue is added. In the presence of specific antibody, the membrane permeability in the parasite is induces by specific antibody so that the cytoplasm can leak out and the tachyzoite does not take up the dye and so appears colorless. In the absence of specific antibody, tachyzoites take up the dye and are stained as blue color. Both sensitivity and specificity of the DT are highly, but this test is only performed in some reference laboratories because it is unsafe due to the use of live parasites. In addition, this test has high costs and needs highly technical expertise in testing.

Indirect hemagglutination test (IHA)

This test is carried out by coating soluble *T. gondii* tachyzoites on tanned red blood cells (RBCs). Then the serum sample is mixed with the RBCs. In the patient who has antibodies against *T. gondii* antigens, the RBCs will agglutinate and show positive result. The IHA test is suitable for detection of *Toxoplasma* infection in humans and animals because it is simple to perform, low costs and practical for testing many serum samples. This test can be highly variable in antigens and in RBC quality. In addition, it can detect antibody later than does the DT (Dubey and Beattie, 1988) and therefore acute infections may be missed for detection of toxoplasmosis (Moghazy *et al*., 2011).

Enzyme-linked immunoabsorbent assay (ELISA)

In the ELISA test, serum sample is added into microtiter plate wells that are previously coated with crude soluble antigen, to form an antigen-antibody complex (if specific antibodies are present). A secondary enzyme-linked antibody specific to the host species is added to enhance the antigen–antibody reaction. The result of the reaction can be evaluated from the color intensity that is measured by an ELISA reader. A color change in the substrate that caused by any attached conjugate is directly associated to the amount of bound antibody. This technique is easy to carry out and can be used for analyzing of large number of serum samples at the same time. Furthermore, most reagents such as substrates, defined anti-species conjugates, and whole kits are commercially available. However, this test requires an ELISA reader and also needs further refinement with regard to procedures and standardization of the antigen used (Gamble *et al.,* 2005). Currently, most clinical laboratories use this method for the routine screening of specific anti-*Toxoplasma* IgG and IgM (Remington *et al.*, 2006).

Modified direct agglutination test (MAT)

The MAT test is performed by mixing antigens (formalin fixed tachyzoites) with 2-mercaptoethanol. Then the mixture is added to serum samples that are placed into microtiter plates. The 2-mercaptoethanol eliminates natural IgM antibodies that result in false positive of agglutination test. This significantly enhances the test specificity. Positive results will present agglutination that can be graded, whereas negative results will present a button of precipitated tachyzoites at the base of the well. This test is simple to perform, but it needs large amount of antigen (Dubey and Desmonts, 1987). The sensitivity and specificity of MAT has been validated by comparing serologic data and isolation of the parasite from naturally and experimentally infected pigs (Dubey *et al*., 1995; Dubey *et al*., 1997). Because the test is both sensitive and specific, and is commonly available as commercial kits, it is the preferred method for the diagnosis of *Toxoplasma* infection in animals.

4.2 Direct detection

4.2.1 Bioassays in mice and cats

Both mice and cats which are the natural hosts of *T. gondii* are used to inoculate and subsequently produce a high number of parasites to facilitate the identification of infection. The advantage of this method does not only allow for multiplication of the parasites, but also can keep the parasites for future studies. However, this technique is time consuming even though it is highly sensitive and specific.

Bioassay in mice

Body fluids (blood, cerebrospinal fluid, aqueous humor, amniotic fluid, etc.) which obtained from suspected samples are taken to process in the laboratory and then it can be inoculated in the mice by intraperitoneal (IP) or subcutaneous injection. After inoculation, the parasite tissue burden may peak within 6 to 10 days post infections. Tachyzoites may be found in the peritoneal fluid, or in the mesenteric lymph nodes and lungs. Mice are euthanized to collect and expand parasites in cell culture. These parasites or mouse homogenate can be cryopreserved in liquid nitrogen for long term storage.

The animal muscular tissues such as tongue, heart, and diaphragm are frequently used to isolate *T. gondii* in mice. Usually, latent infected animals have low amount of *T. gondii* tissue cysts, these cysts are digested by pepsin to liberate parasites and concentrate before infecting mice.

Bioassay in cats

An extremely low number of tissue cysts in animals may not be detectable by bioassay in mice. The use of cats instead of mice can result in an increase of the possibility of parasites isolation because cats can eat much larger volume of tissues (ten times or more) (Dubey and Beattie, 1988). In this method, specific pathogen free cats are fed with more meat samples. All feces should be collected from infected cats to isolate these oocysts. After oocyst sporulation, mice are inoculated with sporulated oocysts by IP injection. During day 7 and 10 post infection, peritoneal lavages are collected from mice for inoculating the tachyzoite in cell culture to expand the parasites for cryopreservation until further tested.

4.2.2 Molecular methods

Several molecular methods based on PCR have been established for the specific detection or analysis of *T. gondii* DNA. Generally, these methods have proved to be easy, sensitive, reproducible and cost-effective, which can be used with various clinical samples both from humans and animals (Bell and Ranford-Cartwright, 2002; Contini *et al*., 2005; Calderaro *et al*., 2006; Bastien *et al*., 2007). However, DNA targets for PCR detection still lack of standardization. Molecular methods can be divided into two categories according to different purposes. The first aim is to confirm the presence of the parasite DNA in biological samples focusing on the high sensitivity of detection methods such as the conventional PCR, nested PCR (nPCR) and quantitative real time PCR (qPCR) of highly repetitive DNA sequences. The second purpose is to identify each *T. gondii* isolate with high resolution using the multilocus genotyping of single copy genes. The molecular technique used in this

purpose consists of the multilocus PCR-RFLP, microsatellite, and multilocus sequence typing (MLST). These methods are essential for epidemiological studies which require to investigate the transmission of *Toxoplasma* infection and to indicate the source of contamination.

4.2.2.1 Conventional PCR, nested PCR (n-PCR) or quantitative real-time PCR (qPCR) of repetitive DNA sequences for detection of *T. gondii*

The detection of *T. gondii* in biological samples frequently uses 2 repetitive DNA sequences as the target including the 35-copy B1 (Burg *et al*., 1989) gene and the 300-copy 529 bp repeat element (Homan *et al*., 2000). Currently, many studies have found that the 529 bp repeat element is higher (10 to 100 times) sensitive than the B1 gene (Costa *et al*., 2000; Reischl *et al*., 2003; Chabbert *et al*., 2004; Hierl *et al*., 2004; Cassaing *et al*., 2006; Edvinsson *et al*., 2006; Menotti *et al*., 2010; Sterkers *et al*., 2010). The 110-copy internal transcribed spacer (ITS1) or 18S rDNA gene sequences are less often used for diagnosis (Hurtado *et al*., 2001; Jauregui *et al*., 2001; Calderaro *et al*., 2006), although their sensitivity is similar to the B1 gene. In comparison with the sensitivity among these PCR techniques, the qPCR is higher sensitive than the n-PCR and the conventional PCR respectively (Contini *et al*., 2005; Okay *et al*., 2009). The qPCR technique is not only detection but also quantification of the parasites. In addition, it also uses to track or control the disease development under drug treatment and to diagnose the low level of infections or carrier states (Contini *et al*., 2005).

4.2.2.2 The multilocus PCR-RFLP, microsatellite, and multilocus sequence typing (MLST) of single copy DNA sequences for characterization of *T. gondii*

In general, these 3 methods are commonly used for genotyping of *T. gondii* including multilocus PCR-RFLP, microsatellite and MLST. The principle of PCR-RFLP relies on the capability of restriction endonucleases to recognize single nucleotide polymorphisms (SNPs), digest PCR products and subsequently exhibit distinct DNA banding patterns on agarose gels by electrophoresis (Howe and Sibley, 1995). Microsatellite analysis is based on the DNA sequence length polymorphisms of short nucleotide tandem repeats. The tandem repeats of *T. gondii* are frequently simple, consisting of as few as 2 nucleotides (di-nucleotide repeats), and take place 2–20 times (Blackston *et al*., 2001; Ajzenberg *et al*., 2002b, Ajzenberg *et al*., 2004). The MLST is based on DNA sequence polymorphisms including the SNPs, insertion and deletion of nucleotides in the sequence. The mutation of microsatellites and SNPs predicts the different rates of evolution. In general, the mutation rate for SNPs in eukaryotes is around at 10^{-9}-10^{-10} per nucleotide position per replication,

whereas microsatellites have the mutation rate about 10^{-2}-10^{-5} per locus per replication (Goldstein and Schlotterer, 1999). Although the mutation rates of SNPs and microsatellite in *T. gondii* remains unknown, the mutation rates of microsatellites are higher than SNPs which can help to increase the resolution of typing (Ajzenberg *et al*., 2002b). The microsatellites benefit for discriminating *T. gondii* isolates that have a close genetic relation. Among all techniques, microsatellite and multilocus PCR-RFLP are simpler and lower costs than MLST, so they are popular approaches for epidemiological studies in order to characterize the *T. gondii* isolates. Because the sensitivity of using a single-copy gene is compromised when compared to a highly repetitive sequence, the sample DNA can be amplified at limited amounts. A multiplex multilocus nested PCR-RFLP (Mn-PCR-RFLP) technique have recently developed using 10 genetic markers and Apico for identification of *T. gondii* isolates in free-range chickens (Dubey *et al*., 2007b). The sensitivity of this method is approximated at 10 *T. gondii* genome equivalents when compared to a previous study with 4 (SAG2, SAG3, BTUB, GRA6) of these markers (Khan *et al*., 2005). Similarly, this method is applied to detect and genotype the parasite strains in cerebrospinal fluid obtained from AIDS patients (Ferreira *et al*., 2008). Based on these results, the Mn-PCR-RFLP typing method with 10 genetic markers has a higher resolution in identification of *T. gondii* isolates obtained from different hosts in various geographical regions (Su *et al*., 2006; Dubey *et al*., 2007c; Pena *et al*., 2008). Moreover, Mn-PCR-RFLP can be performed by using only a few amounts of individual samples.

MATERIALS AND METHODS

1. Determination of seroprevalence of antibodies against *Toxoplasma* infection in pregnant women and HIV patients by ELISA test

1.1 Study site and population

Two groups of people at risk, 1,400 pregnant women and 735 HIV infected patients were recruited in this study. The pregnant women attending the antenatal clinic and 300 HIV patients in any age group visiting the Songklanagarind Hospital, Hat Yai, Songkhla province, Thailand between October, 2009 and June, 2010 were recruited in this study. The remaining 435 HIV infected patients were from the Thammarak Foundation, Phrabatnampu Temple, Lopburi province during May 2011 to January 2012. Their informed consents were obtained from all subjects, and the study was conducted with the approval from the ethical committee of the Faculty of Medicine, Prince of Songkla University, Thailand.

1.2 Collection of blood samples

After informed consent, about 5 mL of venous blood were drawn and serum was collected by centrifugation at $1,100 \times g$ for 5 minutes, then it was kept at -20°C until further used.

1.3 Detection of IgG and IgM antibodies to *T. gondii* (Nissapatorn *et al*., 2011)

Serum samples obtained from pregnant women were screened for anti-*Toxoplasma* IgG and IgM antibodies using a standard commercial ELISA kit (IgG-Trinity Biotech and IgM-Trinity Biotech, New York). The level of anti-*Toxoplasma* IgG and IgM antibodies was obtained and interpreted in accordance with the manufacturer's instructions. The values of anti-*Toxoplasma* IgG antibodies are considered positive when the ISR (immune status ratio) value is equal or greater than 1.10 indicating the current or previous infection. While the results with anti-*Toxoplasma* IgM antibodies value of ≥ 1.10 ISR are considered positive indicating a recently acquired *Toxoplasma* infection. For HIV-infected patients, their serum were also determined the level of anti-*Toxoplasma* IgG and IgM antibodies using a standard commercial ELISA kit (NovaLisa Dietzenbach, Germany). The values of anti-*Toxoplasma* IgG antibodies are considered positive when the IU (international unit) value is greater than 35 indicating the current or past infection. While the results with anti-*Toxoplasma* IgM antibodies value of > 11 NTU (NovaTec-Units) are considered positive indicating a recently acquired infection.

2. Screening for *T. gondii* DNA by PCR method

The anti-*Toxoplasma* IgG and/or IgM positive pregnant women and all HIV infected patients and were taken to perform DNA extraction.

2.1 DNA extraction (Mesquita *et al*., 2010)

Each 200 µL of whole blood samples was added with 20 µl of proteinase K (20 mg/ml; QIAGEN) and AL lysis buffer, and then the mixture was incubated at 56°C for 10 min. After incubation, the QIAamp blood protocol was performed using the QIAamp DNA mini kit (QIAGEN) according to the manufacturer's instructions. The DNA was dissolved in 100 µL of AE buffer (QIAamp DNA Kit) and subsequently stored at -20°C for further analysis.

2.2 Detection of *T. gondii* by PCR analysis targeting the 529 bp repeat element (Homan *et al*., 2000)

A PCR mixture containing 1X PCR buffer free $MgCl_2$, 200 µM concentrations of each deoxynucleoside tri-phosphate (dNTP) (Intron Biotechnology, Korea), 0.2 µM of each primer TOX4 (5'-CGCTGCAGGGAGGAAGACGAAAGTTG-3') and TOX5 (5'-CGCTGCAGACACAGTGCATCTGGATT-3') amplifying a 529 bp fragment of a 200- to 300-fold repetitive DNA fragment, 1.5 mM $MgCl_2$, and 0.5 units of i-*Taq*TM *Plus* DNA Polymerase (Intron Biotechnology, Korea) per 20 µL reactions were prepared; 4 µL of template DNA were used in each reaction for detection of the parasite. The reaction mixture was incubated by one initial denaturation cycle for 5 min at 94°C, 35 cycles of denaturation at 94°C for 45 s, annealing at 58°C for 1 min, and extension at 72°C for 1 min. A final extension step was run at 72°C for 5 min. Positive and negative controls, consisting of a sample containing DNA extracted from tachyzoites and deionized water, respectively, were included in every set of PCRs. The amplification products were electrophoresed on 1.5% TAE (Tris-acetate-EDTA) agarose gel stained with ethidium bromide, and visualized under UV light. A standard 100-bp DNA size marker was included in every gel.

3. Evaluation of the prevalence of *T. gondii* tissue cysts in meat samples

3.1 Sample collection

The samples were collected from 3 local meat retailers (the Plaza Market, Khlong Rean Market and Kho Mee Market) in Hat Yai city, Songkhla province including: 40 pork and beef samples (20 samples each type). One hundred grams of 5 different parts, including the brain, heart, liver, lung and tenderloin tissue samples, of each animal species were perchased from those retailers, and 50 grams of each collected sample were cut into a

smaller size with a sterile knife. Then the sample was ground with liquid nitrogen using a mortar and pestle rinsed with hot water to prevent cross-contamination. The ground tissue sample was taken to use in a next step of DNA extraction.

3.2 DNA extraction

A total of 500 mg of each minced tissue were used for DNA extraction using a commercial DNA extraction kit (Invitrogen, CA, USA) according to the manufacturer's instructions. Briefly, 500 mg of ground tissue samples were digested with 900 μL ATL buffer and 100 μL proteinase K (supplied in the QIAamp Stool DNA extraction Kit) overnight on a shaker at 56°C. Subsequently, 200 μL of digested tissue were further processed with the standard protocol, according to the manufacturer's instructions. All DNA extracts were frozen at -20°C until used. This product was used as a template for PCR.

3.3 Detection of *T. gondii* by PCR analysis targeting the 529 bp repeat element (Homan *et al*., 2000)

The DNA extracted from digested tissue samples was taken to amplify the 529 bp repeat element as the same method to Section 2.2.

4. Genotyping of *T. gondii* by multiplex multilocus nested PCR-RFLP (Mn-PCR-RFLP) technique (Dubey *et al*., 2006; Su *et al*., 2010)

The strain typing of *T. gondii* genomic DNA was performed using genetic markers *SAG1, SAG2*-new, *SAG3, BTUB* and *GRA6* as described previously (Grigg *et al*., 2001; Khan *et al*., 2005; Su *et al*., 2006) with modification (Table 1). Briefly, the target DNA sequences were firstly amplified by multiplex PCR using external primers for all five markers. The reaction was carried out in 25 μL of volume containing 1X PCR buffer, 2 mM $MgCl_2$, 200 μM each of the dNTPs, 0.2 μM each of the forward and reverse primers, 0.5 units of 0.5 units of i-*Taq*^{TM} *Plus* DNA Polymerase (Intron Biotechnology, Korea) and 5 μL of extracted DNA.

The reaction mixture was firstly heated at 94°C for 5 min, followed by 35 cycles of 94°C for 45 s, 55°C for 1 min and 72°C for 1 min. A last extension cycle was run at 72°C for 5 min. Multiplex PCR amplified products (5 μL) were then used for nested PCR amplification (35 cycles) with internal primers for each marker separately, using an annealing temperature of 60°C (for *SAG1* and *SAG2*-new) or 62°C (for *SAG3, BTUB* and *GRA6*) for 1 min in 25 μL volume reaction mixture as above. The nested PCR products were resolved in a 1.5% agarose gel by electrophoresis, stained with ethidium bromide and

visualized under UV light. A standard 100-bp DNA size marker was run in every gel to estimate the size of amplified fragments.

To reveal the digestion pattern, 5 μL of nested PCR products were properly incubated with the digestion reaction containing restriction enzymes for each gene *(SAG1*: *Sau96*I and *Hae*II; *SAG2*-new*: Hinf*I and *Taq*I*; SAG3: Nci*I; *BTUB: BsiE*I and *Taq*I; and *GRA6*: *Mse*I), following the protocols recommended by the manufacturer (Thermo Scientific Fast Digest, USA). The digested PCR products were visualized after separating on a 2.5% agarose gel by electrophoresis and staining with ethidium bromide. Three genomic DNA of *T. gondii* strains: GT1 (type I), PTG (type II) and CTG (type III) were served as standard reference strains for *T. gondii* genotyping, and deionized water was used as a negative control which was also included in every set of PCRs.

Table 1 Summary of five genetic markers used for genotyping of *T. gondii*.

Markers	External primer (for multiplex PCR)*	Internal primers (for nested PCR)	Restriction enzymes	reference
SAG1	F: GTTCTAACCACGCACCCTGAG	F: CAATGTGCACCTGTAGGAAGC	Sau96I+HaeII (double digest)	Grigg *et al*., 2001
	R: AAGAGTGGGAGGCTCTGTGA	R: GTGGTTCTCCGTCGGTGTGAG		Dubey *et al*., 2006
SAG2-new	F: GGAACGCGAACAATGAGTTT	F: ACCCATCTGCGAAGAAAACG	HinfI+TaqI (double digest)	Su *et al*., 2006
	R: GCACTGTTGTCCAGGGTTTT	R: ATTTCGACCAGCGGGAGCAC		
SAG3	F: CAACTCTCACCATTCCACCC	F: TCTTGTCGGGTGTTCACTCA	NciI	Grigg *et al*., 2001
	R: GCGCGTTGTTAGACAAGACA	R: CACAAGGAGACCGAGAAGGA		
BTUB	F: TCCAAAATGAGAGAAATCGT	F: GAGGTCATCTCGGACGAACA	BsiEI+TaqI (double digest)	Khan *et al*., 2005
	R: AAATTGAAATGACGGAAGAA	R: TTGTAGGAACACCCGGACGC		Dubey *et al*., 2006
GRA6	F: ATTTGTGTTTCCGAGCAGGT	F: TTTCCGAGCAGGTGACCT	MseI	Khan *et al*., 2005
	R: GCACCTTCGCTTGTGGTT	R: TCGCCGAAGAGTTGACATAG		

* F = forward primer; R = reverse primer

RESULTS

1. Seroprevalence of *T. gondii* infections in pregnant women and HIV infected patients

The prevalence of *Toxoplasma* infection in 1,400 pregnant women and 735 HIV infected patients tested by ELISA method was shown in Table 1. The overall seroprevalence of this infection in pregnant women and HIV subjects was 30.9% (432/1400), and 27.5% (202/735) respectively. In pregnant women, 305 (21.8%) were positive for only *Toxoplasma*-specific IgG antibodies, and 61 (4.4%) patients were positive for only *Toxoplasma*-specific IgM antibodies. Additionally, 66 (4.7%) were positive for both *Toxoplasma*-specific IgG and IgM antibodies. The *Toxoplasma* seropositivity in HIV infected patients was 194 (26.4%) cases for only *Toxoplasma*-specific IgG antibodies, and 6 (0.8%) cases for only *Toxoplasma*-specific IgM antibodies. Of these, 2 (0.3%) cases were also positive for both *Toxoplasma*-specific IgG and IgM antibodies.

Table 2 Anti-*Toxoplasma* IgG, IgM in pregnant women and HIV infected patients determined by with ELISA test

Sample group	No. (%) of positive samples for anti-*Toxoplasma* IgG, IgM			
	IgG (+), IgM (-)	IgG (-), IgM (+)	IgG (+), IgM (+)	Total
Pregnant women (n=1400)	305 (21.8)	61 (4.4)	66 (4.7)	432 (30.9)
HIV infected patients (n=735)	194 (26.4)	6 (0.8)	2 (0.3)	202 (27.5)

2. Screening for *T. gondii* DNA by PCR analysis targeting the 529 bp repeat element

Four hundred and thirty two DNA samples extracted from pregnant women who were positive for anti-*Toxoplasma* IgG and/or IgM antibodies, and 735 DNA samples extracted from HIV-infected patients were not PCR-positive for *Toxoplasma* DNA with the 529 bp repeat element.

3. Evaluation of the prevalence of *T. gondii* tissue cysts in meat samples

None of 200 tissue samples (brain, heart, liver, lung, and meat) collected from cattle and pigs were positive for *Toxoplasma* tissue cysts when tested by PCR analysis targeting the 529 bp repeat element.

4. Genotyping of *T. gondii* by multiplex multilocus nested PCR-RFLP (Mn-PCR-RFLP) technique

Since we could not detect any *Toxoplasma* DNA from blood or meat samples, we therefore extracted DNA from some cat feces to perform genotyping and found that 8 of 13 *T. gondii* positive samples were successfully amplified with one or more genetic markers. Only 2 samples (no. 45 and 74) were amplified using all five markers, while one sample (no. 49) could be amplified with *SAG1, SAG3* and *BTUB* markers (Figure 2). The results of digested PCR products and the RFLP patterns of five genetic markers demonstrated that one (7.7%) isolate was type I, two (15.4%) isolates were type III and one (7.7%) isolate was type II or type III. The combinations of different alleles were also presented in 4 (30.7%) isolates of *T. gondii* which these were classified as mixed genotypes (Figure 2 and Table 3).
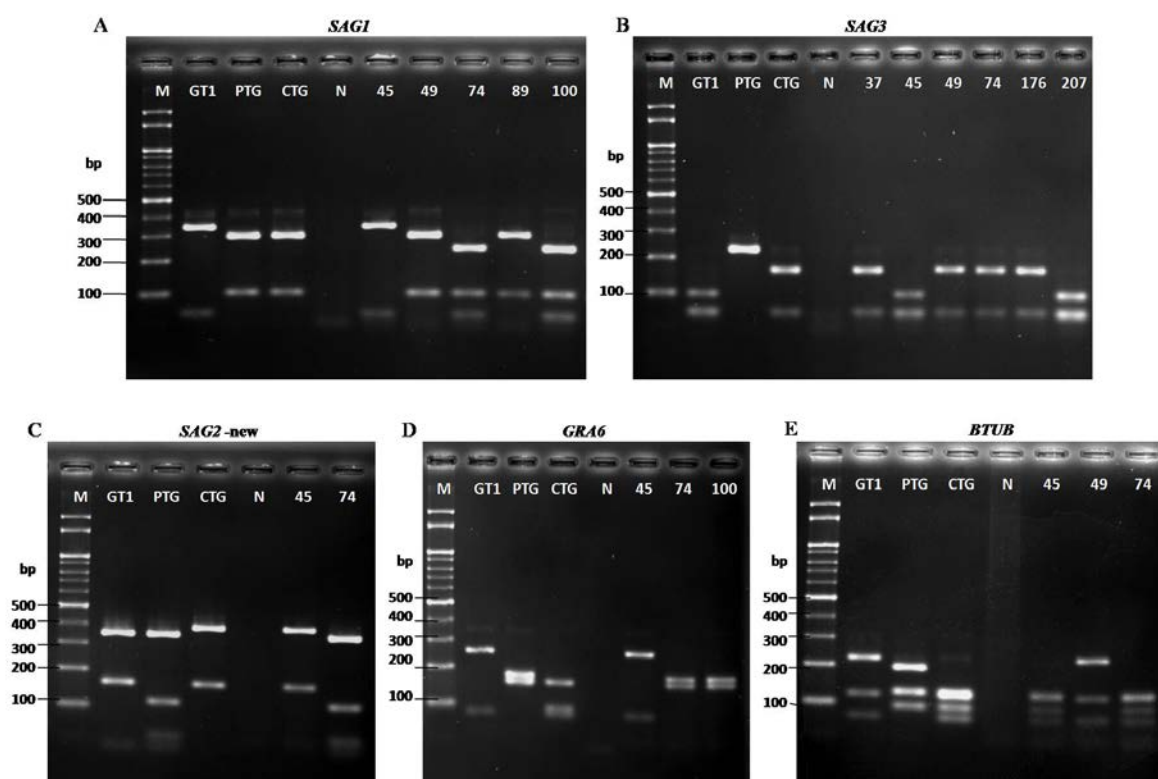
Figure 2 The PCR-RFLP analysis of the *SAG1* (A), *SAG3* (B), *SAG2*-new (C), *GRA6* (D) and *BTUB* (E) gene from 8 *T. gondii* positive samples. GT1 (type I), PTG (type II), and CTG (III) were used as *T. gondii* reference strains. All markers were resolved by electrophoresis on 2.5% agarose gel stained with ethidium bromide. Lane: M, 100 bp molecular weight marker; lane: N, negative control; lanes: 37, 45, 49, 74, 89, 100, 176 and 207; the digested PCR products of feces sample no. 37, 45, 49, 74, 89, 100, 176 and 207 respectively.

Table 3 Summary of multilocus genotyping of *T. gondii* isolated from cat feces samples

Strains/ sample no.	Allele type at each markers					Genotypes
	SAG1[a]	*SAG2*-new	*SAG3*	*BTUB*	*GRA6*	
GT1	I	I	I	I	I	I
PTG	II/III	II	II	II	II	II
CTG	II/III	III	III	III	III	III
37	-[b]	-	III	-	-	III
45	I	I	I	III	I	Mixed
49	II/III	-	III	I	-	Mixed
74	u-1[c]	II	III	III	II	Mixed
89	II/III	-	-	-	-	II/III
100	u-1	-	-	-	II	Mixed
176	-	-	III	-	-	III
207	-	-	I	-	-	I

[a] allele type II and III were similar.

[b] -, negative amplification product

[c] u-1 was atypical alleles which different from major clonal Type I, II and III alleles.

DISCUSSION

In this study, the overall serological positive rates of *Toxoplasma* infections among pregnant women were found 30.9% which were higher than prevalence rates of previous reports from China 10.6% (Liu *et al.,* 2009), USA 11% (Pappas *et al*., 2009) and Japan 10.3% (Sakikawa *et al*., 2012). Most of pregnant women tested were positive for only anti-*Toxoplasma* IgG antibodies (21.8%) which indicated a previous infection due to the past exposure to *T. gondii*, and their fetuses were little or no risk of acquiring congenital toxoplasmosis except in mothers who become infected up to 3 months before conception (Vogel *et al.*, 1996; Gavinet *et al.*, 1997). The risk of mother-to-child transmission and the severity of congenital damage vary depending on the gestational age at maternal infection (Dunn *et al*., 1999). Once this infections occur in pregnant women during the first trimester, the risk of congenital infection decreases (10-15%), whereas it highly increases (60-90%) in the third trimester (Dunn *et al*., 1999). On the contrary, the severity of disease is the highest in the first trimester and lowest in the third trimester (Holliman, 1995; Remington *et al*., 2006). In a group of 4.4% pregnant women with a positive serology for only anti-*Toxoplasma* IgM antibodies could be indicated a recently acquired infection; however, false positive results of anti-*Toxoplasma* IgM antibodies cannot be excluded because these specific IgM antibodies can be detected for long time over one year after acute infection (Wilson and McAuley, 1999). The second serum specimen is needed to be collected from the same patients at 2-4 weeks after the first to follow up the rising antibody titer of IgG antibodies. If the results of the second serum are positive with IgM antibodies but negative with IgG antibodies, the IgM result should be considered as a false positive reaction and the patients are not infected (Montoya and Liesenfeld, 2004; Meroni and Genco, 2008). In the cases of pregnant women (4.7%) who were positive for both *Toxoplasma*-specific IgG and IgM antibodies, the result may indicate past or recently acquired infection. This patient group is necessary to be followed up the IgG and IgM results from the second serum specimen to confirm the exact time of infection, and both first and second serum samples may be sent to *Toxoplasma* reference laboratory to diagnose and confirm the results (Montoya and Remington, 2008; Tekkesin, 2012, Paquet and Yudin, 2013). The present study also found 69.14% (968/1400) of pregnant women tested negative with both IgG and IgM against *T. gondii*, which indicated that they have not been exposed to the parasite. Nevertheless, these patients are highly susceptible to contract *Toxoplasma* infections in the future. *Toxoplasma* infections in pregnant women have been reported variously from many

countries. The high incidence of *Toxoplasma* positive serology during pregnancy most commonly found in European countries (Cook *et al.*, 2000; Berger *et al.*, 2009) and in the South America (Rebouças *et al.*, 2011), whereas a low prevalent rate of this infection was found in Asian countries (Han *et al.*, 2008; Buchy *et al.*, 2003) except for India (Singh and Pandit, 2004; Borkakoty *et al.*, 2007) and Malaysia (Nissapatorn *et al.*, 2003). The varied prevalence rates may possibly be due to the difference in diagnostic method used (Chemoh *et al.*, 2013) or the different climatic conditions in each area that might affect the ability of *T. gondii* oocysts to survive in natural environments (Kistiah *et al.*, 2011). In addition, the variations in seroprevalences of *Toxoplasma* infections were related to individual's eating and cultural habits (Robert-Gangneux and Dardé, 2012).

Toxoplasmosis is one of opportunistic infections that are frequently found in patients infected with HIV, and it is estimated that half of those are infected with *T. gondii* (Shimelis *et al,* 2009; Daryani *et al.,* 2011). The seroprevalence of this infection among Thai HIV-infected patients has been reported from previous studies, and the infection rates ranged from 22.4%-53.7% (Wongkamchai *et al.,* 1995; Chintana *et al.,* 1998; Sukthana *et al*., 2000; Nissapatorn *et al.,* 2001; Wanachiwanawin *et al.,* 2001). In our results, the seropositivity of only anti-*Toxoplasma* IgG antibodies in HIV positive study participants was 26.4% which indicated a past infection with the parasite. The presence of *Toxoplasma* specific IgG antibodies in these cases was the outcome of a reactivation of a latent infection that may develop the manifestations of diseases into toxoplasmic encephalitis (TE), particularly when their CD_4^+ T-cell count decreases less than 100 cells/µL (George *et al.*, 2009; Luma *et al.*, 2013). Therefore, the screening of anti-*Toxoplasma* IgG antibodies using serological methods should be carried out in all HIV-infected patients to find out the risk of developing TE (Jayawardena *et al.*, 2008; Daryani *et al.*, 2011). The present study was also found that six (0.8%) cases of HIV-infected patients had anti-*Toxoplasma* IgM antibodies. This lower rates of IgM seropositvity is related to other previous studies: 1% in Mexico (Ramírez *et al.*, 1997) and Iran (Mohraz *et al.*, 2011), 1.8% in Nigeria (Ogoina *et al*., 2013). The seroconversion of anti-*Toxoplasma* IgM antibodies cannot be diagnosed an acute toxoplasmosis in patients with HIV, since the the specific IgM antibodies are usually not present in patients with reactivated disease (Benson *et al.*, 2004).

Our study was not detected any *Toxoplasma* DNA from blood tested samples belonging to seropositive pregnant women and all HIV-infected patients using PCR analysis. The PCR techniques have been applied for the specific detection of *T. gondii* DNA in various clinical samples both from humans and animals (Contini *et al*., 2005; Calderaro *et*

al., 2006; Bastien *et al*., 2007). These methods have proved to be easy, sensitive, reproducible and cost-effective, and also be used in the noninvasive diagnosis of TE in AIDS patients with high specificity (Bretagne, 2003; Colombo *et al*., 2005; Mesquita *et al*., 2010). Currently, many studies frequently use the 529 bp repeat element as the target for amplification of *Toxoplasma* DNA because the sensitivity is higher than B1 gene (Reischl *et al*., 2003; Chabbert *et al*., 2004; Cassaing *et al*., 2006; Menotti *et al*., 2010). In this study, the PCR technique was performed in all patients with HIV because the serological tests could not be considered as a definitive diagnostic method for identifying the presence of *Toxoplasma* DNA in patients with deficient immunity (Montoya and Liesenfeld, 2004). Moreover, it has been reported that 13.3% of HIV-infected patients with negative for anti-*Toxoplasma* antibodies showed positive results with PCR detection (Lindström *et al.*, 2006). Guy and Joynson (1995) has been found that the *Toxoplasma* parasitemia during acute phase of infection was positively identified by PCR from the second week to the 17th weeks of the initial infection, but the positive results for serological tests in patients were not essential to correlate with an acute phase of infection (Haghpanah *et al.*, 2006). Therefore, the failure for detection of *Toxoplasma* DNA in all tested samples was able to confirm the past infection more than four months ago.

Eating of raw or undercooked meat containing *T. gondii* tissue cysts is one of important routes of acquiring *Toxoplasma* infection in humans (Cook *et al.*, 2000; Kravetz and Federman, 2005). Pork meat is commonly consumed in many countries, although it has been previously regarded as a main source that responsible for human *Toxoplasma* infection (Dubey, 2009). In Thailand, the consumption of pork is higher than beef meat that may be explained by the cheaper price of pork than beef (FAO, 2005). In this study, we evaluated the prevalence of *T. gondii* tissue cysts in 2 types of meat samples: pork and beef from retail meat stores using PCR analysis. The tissue cysts of *T. gondii* were not detected in any 5 different parts of both meat samples. These findings indicate that pork and beef may not a source of infection in human toxoplasmosis in the studied area. However, the negative results may be caused by other possible three reasons (i) the random distribution of the tissue cysts within each tissue, (ii) a small number of *T. gondii* tissue cysts, (iii) the presence of this parasite in unexamined parts of tissue samples (Opsteegh *et al.*, 2011; Glor *et al.*, 2013; Boughattas *et al.*, 2014). Generally, the tissue cysts may exist throughout life of the infected hosts; however, the number of tissue cysts and the locations where the parasite like to live differ in a certain species of intermediate host (Dubey, 2000). The tissue cysts are often localized in the central nervous system, the skeletal and cardiac muscles. In addition, visceral

organs such as lungs, liver and kidney are also found to be the predilection site of the tissue cysts (Dubey, 1993; Dubey *et al.*, 1998). The result observed in this study was not consistent with several previous studies which showed that the prevalence rate of *T. gondii* tissue cyst in pork was 2.2% in Switzerland (Berger-Schoch *et al.*, 2011), 18.03% in China (Wang *et al.*, 2012), and in beef cattle was 1% in Brazil (Santos *et al.*, 2010), 4.7% in Switzerland (Berger-Schoch *et al.*, 2011). In comparison to a recent study conducted in Thailand which reported the prevalence of *Toxoplasma* tissue cyts in 1.38% of minced pork collected from selected markets (Sutthikornchai *et al.*, 2013). It is possible that differences in method used, also the sample size explain the different prevalence of *Toxoplasma* tissue cysts found in our study. Sutthikornchai *et al*. had selected the quantitative real-time PCR for detection of *T. gondii*, and the sample size of pork meat tested was 100 grams per sample, whereas we carried out the qualitative PCR and the sample size of each different tissue from each animal was 50 grams. Moreover, various types of tissue samples including brain, heart, liver, lung and tenderloin tissue samples were used in our study which this sampling can increase the chance of *Toxoplasma* detection (Rahdar *et al*., 2012; Glor *et al.*, 2013).

The genotypic analysis of *T. gondii* from animals and humans has been reported in many areas (Pena *et al.*, 2006; Ferreira *et al.*, 2008; Herrmann *et al.*, 2014). The major clonal types (I, II, and III) were predominantly found in Europe and North America, while other non-clonal types or mixed infections were widely distributed in South America, Africa and Asia (Khan *et al*., 2009, 2011). In Thailand, there is no study on *T. gondii* genotyping; even though, the seroprevalence of *Toxoplasma* infections has been reported in some previous researches. In this study, *Toxoplasma* DNA was not found in any tested samples collected from blood and meat samples, we therefore need to determine *T. gondii* from some cat feces to investigate the direct genotyping of this parasite. Our results showed that 13 (5.1%) of 254 cat feces samples collected from cat's owners and veterinarians in Songkhla Province were positive for *Toxoplasma* oocyst DNA. These positive isolates were characterized by PCR-RFLP with the *SAG1*, *SAG2*-new, *SAG3*, *BTUB* and *GRA* genes. The majority (30.7%) of our *T. gondii* isolates were mixed genotypes with a combination of different alleles. Interestingly, 2 samples (sample no. 74 and 100) were observed a unique allele (u-1) at *SAG1* locus. These mixed isolates were resulted of genetic exchange which could occur when a cat was infected with more than one different type of *T. gondii* isolates from their intermediate hosts at almost the same period (Herrmann *et al*., 2010; Ferreira *et al*., 2008). The recombination of *T. gondii* genes can also occur randomly in the host that this contributes to possible findings of more different genotypes sharing the same alleles

(Ajzenberg *et al.*, 2004; Khan *et al.*, 2005; Dubey *et al.*, 2007b). The evidences of experimental mixed infections with two strains of distinct genotypes have been previously reported in mice (Dao *et al.*, 2001) and cats (Saeij *et al.*, 2006; Taylor *et al.*, 2006). In our present study, there were some *T. gondii* isolates that could not be amplified with two or all tested markers. This finding was described by a very low amount of the parasite DNA presented in those samples. Several studies have been used bioassays in mice or cats to increase the sufficient concentration of the parasite DNA before performing the genotyping with PCR-RFLP technique (Pena *et al.*, 2006; Bezerra *et al.*, 2012; Herrmann *et al.*, 2010), but this can cause a false prevalence of *T. gondii* strains due to unreal selection in relation to the culture process (Fuentes *et al.*, 2001; Gallego *et al.*, 2006).

The present study is the first report of genotypic characteristics of *T. gondii* isolated from naturally infected animals in Thailand which our results indicate that the parasite oocysts may be the original source that cause contamination and distribution of this parasite into environments mainly through soil and water, and provide high risks for acquisition by humans of *Toxoplasma* infections.

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CONCLUSION

The overall seroprevalence of *Toxoplasma* infections among 1,400 pregnant women and 735 HIV-infected patients tested in this study was 30.9% and 27.5% respectively. Most of both patient groups were indicated as previous infection due to past exposure to *T. gondii* as shown by anti-*Toxoplasma* IgG antibodies, few of them were in an acute infection (anti-IgM antibodies). PCR analysis targeting the 529 bp repeated element from all HIV-infected patient and 432 *Toxoplasma* seropositive pregnant women were negative for *Toxoplasma* DNA. The negative results obtained from meat samples may imply that the ingestion of pork or beef may not the source of *Toxoplasma* infections in humans in the studied area. The PCR-RFLP patterns of *SAG1, SAG2*-new, *SAG3, BTUB* and *GRA6* markers from cat feces revealed type I, II, III and mixed genotypes of alleles at different loci. Whether or not the oocysts from cat feces play an importance role in *Toxoplasma* infection still need more evaluation.

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APPENDIX

1. Reprint

Chemoh, W., Sawangjaroen, N., Nissapatorn, V., Suwanrath, C., Chandeying, V., Hortiwakul, T., Andiappan, H., Sermwittayawong, N., Charoenmak, B., Siripaitoon, P., Lekkla, A. and Sukthana, Y. 2013. *Toxoplasma gondii* infection: What is the real situation? Experimental Parasitology 135: 685-689.

2. Manuscript

Title: Investigation of *Toxoplasma gondii* oocysts in cat feces using the molecular diagnosis of the TOX element and ITS-1 region