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Quantitative PCR detection of *Toxoplasma gondii* in minced pork from selected morning markets in Bangkok, Thailand

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Abstract

Pigs are one of the intermediate hosts of *Toxoplasma gondii*. The parasite has an asexual development cycle in pigs that results in tissue cysts containing bradyzoites, which remain in the hosts for their entire lifespan, making them a source of infection to new hosts, including humans. Our study investigates whether pigs are a low-risk source of *T. gondii* infection or if it is under-recognized, by using a real-time PCR assay to examine and quantify *T. gondii* contamination in 145 minced pork samples obtained from 43 retail meat shops from Thawi Watthana, Phaya Thai, Don Mueang, Nong Chok, and Bang Khun Thian districts of the Bangkok Metropolitan Area. Two of 145 (1.38%) samples were positive and contained 33,100 and 21,900 *T. gondii* bradyzoites per 100g of minced pork. This high parasite load surpasses that of an infective dose of *T. gondii*, prompting an urgent need for health education for consumers.

Keywords: quantitative PCR, minced pork, *Toxoplasma gondii*, contamination, Bangkok Thailand

Introduction

Toxoplasma gondii is a sexually reproductive protozoan that occurs only in domestic and wild Felidae, its definitive hosts, resulting in the shedding of the infective stage, the oocyst. Warm-blooded animals, including birds, rodents, and humans, are intermediate hosts where tachyzoites and bradyzoites multiply. The bradyzoite is a slowly replicating form within tissue cysts [1], which can be spread to new hosts, including humans. Two main transmission routes exist, 1) consuming contaminated food and water with oocysts from cat's feces, and 2) ingestion of undercooked meat containing tissue cysts.

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Transplacental transmission can also occur, but is the least common route [2,3].

Pork is a dominant food source in many countries, including Thailand. From 1997 to 2005, global pork consumption increased by 27%, with the total world pork consumption for 2005 at >93 million tons [4]. Denmark ranked first in world pork consumption, with 73.7 kg per capita while two countries in Asia – Hong Kong and Taiwan – ranked 6th and 9th (54.3 and 42.5 kg per capita, respectively). Consumption in other Asian countries has also been rising steadily – in 2007 the levels were as follows: South Korea (20.9 kg per capita), Japan (17.0 kg per capita), Singapore (13.9 kg per capita), the Philippines (12.5 kg per capita) and Thailand (13.7 kg per capita). This trend has been increasing since 1999 [5].

T. gondii causes morbidity and mortality in

both pigs and humans, and particularly affects neonatal pigs. Most pigs acquire the infection post-natally by the ingestion of oocysts from a contaminated environment, or by the ingestion of an infected animal [6]. Due to modern industrialized pig production and good global husbandry management systems, the prevalence of *T. gondii* in pork has declined steadily, from about 23-42% to 0.9-28.8%. However, in poorly managed non-confinement farms, the prevalence has been as high as 68% [6-8]. Moreover, Kijlstra *et al.* demonstrated that even in areas of low prevalence, 2.3% of free-range pig production systems in the Netherlands had *T. gondii* antibodies [9-11]. Several studies from various parts of China indicate that *T. gondii* seroprevalence in pigs was 24.5-58.1%, and was particularly common in older pigs [12-14]. Information on toxoplasmosis among pigs in Thailand is limited; one study showed a prevalence of *T. gondii* as low as 7.8% [15]. However, an outbreak in one farm resulting in 26.1% morbidity and 4.4% mortality was reported in 2006 [16].

Prangé *et al.* [17] reported the global *T. gondii* seroprevalence in pork samples was 8.8% (range 8.2-9.37). On the other hand, 9.6-92.7% of viable

T. gondii were isolated from many tissues, such as the tongue, diaphragm, heart, and brain, of naturally infected pigs in the USA [6]. In Brazil, 8.7-27.1% of pork sausage and salami were also found contaminated with *T. gondii* [6,18]. These varying contamination levels might be explained by the fact that different studies used different detection methods and sometimes insensitive procedures. The current detection methods for *T. gondii* in meat-producing animals and meat products are inadequate, since they provide only indirect or qualitative data from which it is difficult to assess the true risk of infection. The aim of this study was to obtain accurate information as to whether pigs are a low-risk source of toxoplasmosis or whether it is under-recognized in Thailand. We conducted a study to evaluate and quantify the contamination of *T. gondii* in pork meat from retail markets in the Bangkok Metropolitan area by using real-time PCR technique.

Materials and methods

Sample collection

Of the 50 districts of the Bangkok Metropolitan area (Figure 1), we selected the five districts containing the biggest markets for purchasing



Fig 1 Study locations in 5 districts at the periphery of Bangkok Metropolitan area, in the North, South, West, and East (Assigned as 1-5: 1= Thawi Watthana, 2= Phaya Thai, 3= Don Mueang, 4= Nong Chok, 5= Bang Khun Thian)

minced pork samples. These districts were Thawi Watthana, Phaya Thai, Don Mueang, Nong Chok, and Bang Khun Thian. Three times every alternate day, 200g samples of minced pork were bought from all shops in the fresh morning markets. In total, 145 minced-pork samples were examined in this study; 38 from 12 shops in Bang Khun Thian, 30 from 10 shops in Don Mueang, 30 from 10 shops in Thawi Watthana, 25 from 8 shops in Phaya Thai, and 22 were from 3 shops in Nong Chok District, which is known as a Muslim area with few pork shops.

Preparation of pork samples for detection of *T. gondii*

All meat samples were kept at -80°C until the time of analysis. The process of *T. gondii* detection was adapted from Dubey [19]. A 100 g sample was digested individually in a plastic beaker with pepsin solution (pepsin 2.6 g, NaCl 5.0 g, HCl 7.0 ml and distilled water 500 ml, pH 1.10-1.20) for 1 hour. Next, the suspension was incubated at 37°C in a shaking incubator for 1 hour. The homogenate was filtered through two layers of gauze into a 250 ml centrifuge bottle and centrifuged at 1,200 g for 10 min; the supernatant was then discarded. The sediment was pooled in a 50 ml conical tube, re-suspended in 20 ml of PBS, and neutralized with 12-15 ml of 1.2% sodium bicarbonate (pH 8.3). Centrifugation was performed at 1,200 g for 10 min; most of the supernatant (about 16 ml) was discarded, but 4 ml of the solution was kept and stored at 4°C for DNA extraction.

DNA extraction

Two ml of processed pork suspension were transferred to a micro tube and centrifuged at 1,200 g for 10 min. The supernatant was discarded. The pellets were used for DNA extraction by NucleoSpin® Tissue (Macherey-Nagel, Germany), according to the manufacturer's instructions. DNA was eluted with 100 µl TE buffer and kept at -20°C until used.

Real-time PCR reaction

The 35-copy B1 gene was used to detect

possible pork contamination with *T. gondii*. Primers were newly designed at the strongly conserved regions of many *T. gondii* strains using ClustalW2 (available at: <http://www.ebi.ac.uk/Tools/msa/clustalw2/>). The forward, reverse, and probe were; B1F 5'-GGAAGAGATCCAGCAGATCTCGT-3', B1A 5'-CAGCAAAGAATGTTGCATT-3', and B1probe 5'-6FAM-CCGCCCCACAAGACGGCT-BBQ-3'. The selected primers and probes were aligned against known databases, using BLASTN, to rule out non-specific binding to human and other pathogens. The real-time PCR was composed of 2 µl of sample DNA, 0.4 µM of each primer, 0.2 µM of probe and 10 µl SensiMix™ Probe Kit (Bioline Ltd, UK) and deionized water to make a 20 µl reaction. The PCR program comprised a first denaturation at 95°C for 10 min, followed by 45 cycles at 95°C for 10 s and 59°C for 45 s. Reactions were performed with a Corbett Rotor-Gene™ 6000 (QIAGEN, Australia). The positive DNA control was obtained from RH-strain *T. gondii* tachyzoites cultured in mice at the Department of Protozoology, Faculty of Tropical Medicine, Mahidol University. The lower limit of detection for the real-time PCR was tested with *T. gondii* DNA mixed with DNA extracted from negative minced pork. The amplification curve specific to *T. gondii* appeared at the 23rd cycle (Figure 2 A) and amplification of a 10-fold dilution (from 1-10⁵ cell) showed the lowest limit of detection to be 1 cell/20 µl reaction, with correlation coefficient (R) = 0.99720 (Figure 2B).

Each extracted meat sample was examined twice for *T. gondii* contamination by real-time PCR assay using the primers and Taqman probes, as mentioned above. Then, all positive samples were further quantified to *Toxoplasma* contamination levels with a 10-fold dilution curve.

Data collection

Information about each pork sample, e.g. including number, market, and date purchased, were recorded. Data for the positive and negative contamination of *T. gondii* in the pork samples were collected from the amplification curve according to the threshold cycle (Ct). The quantity

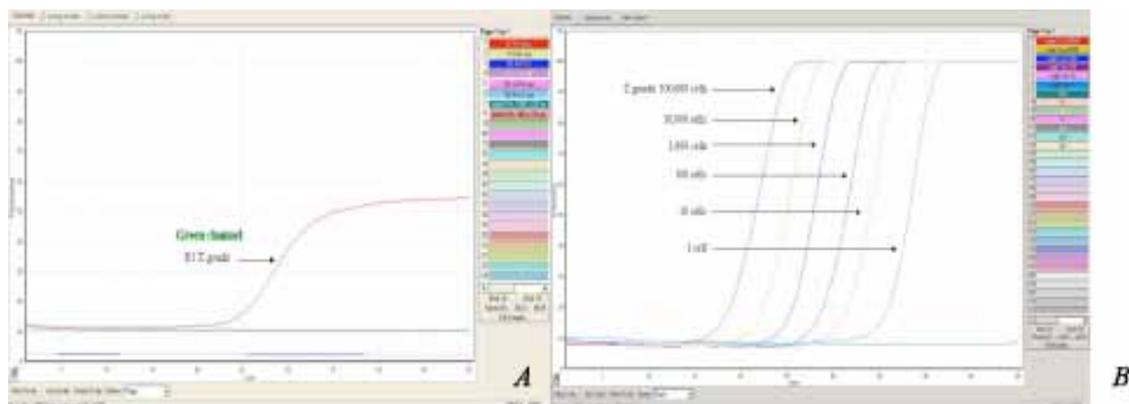


Fig 2 Real-time PCR assay showed (A) amplification curve specific to *T. gondii* by using B1 gene primer with Taqman probe and (B) amplification of a 10-fold dilution showing the lowest limit of detection at 1 cell/20 ml reaction (correlation coefficient (R) = 0.99720)

of *T. gondii* contamination in a positive sample was calculated by the standard curve, and reported as the number of tachyzoites/bradyzoites per 100 g of minced pork.

Results

As few as 10 tachyzoites/bradyzoites *T. gondii* DNA ($R^2 = 0.999$, $E = 0.91$) per 20 μ l reaction were detected. Two of 145 (1.38%) samples from Nong Chok (S1/4A) and Bang Khun Thian (S18/5C) districts were positive for *T. gondii* at Ct 32.91 and 32.28, respectively. The positive samples were detected between concentrations of 10^2 and 10^3 *T. gondii* standard curve (S1/4A at Ct = 32.91 and S18/5C at Ct = 32.28), as shown in Figure 3. Using the formula $y = ax + b$, sample S1/4A contained approximately 219 tachyzoites/bradyzoites, while the S18/5C sample was contaminated with 331 tachyzoites/bradyzoites ($R^2 = 0.988$, $E = 0.93$) per 20 μ l reaction. When we calculated the numbers of parasites per 100 g of pork, we found that the samples S1/4A and S18/5C contained 21,900 and 33,100 tachyzoites/bradyzoites per 100 g of minced pork.

Discussion

We studied the contamination and quantification of *T. gondii* in minced pork from retail markets in the Bangkok Metropolitan

area and found it was as low as 1.38%. The low prevalence may be explained by the sampling technique. Specific pig organs such as the heart, tongue, diaphragm, and brain have been reported to contain more tissue cysts than skeletal muscle [6,20]. It is thus difficult to detect *T. gondii* in skeletal tissues of large animals due to the nonspecific sites preferred by the parasite, resulting in the possibility of false-negative results [21]. Mouse or cat bioassay is a reference method for the isolation of *Toxoplasma* from meat products but requires live animals, which is time-consuming and unsuitable for slaughterhouse testing, as well as undesirable from an ethics point of view [22,23]. We attempted to overcome this limitation by using minced pork prepared from meat from various parts of the pig's body – thereby increasing the chance of *T. gondii* detection. In addition, we selected retail markets in the periphery of the city because in those areas there are fewer pigs from bio-secure housing systems than in the inner-city area to increase the chance of detecting contamination. Although, most pork meat in retail markets of Bangkok were from legal slaughterhouses and farms in Nakhon Pathom Province, a major pig producer area of Thailand. There was no information regarding the original sources or farm-management systems since these slaughter pigs might come from any farms in the Central

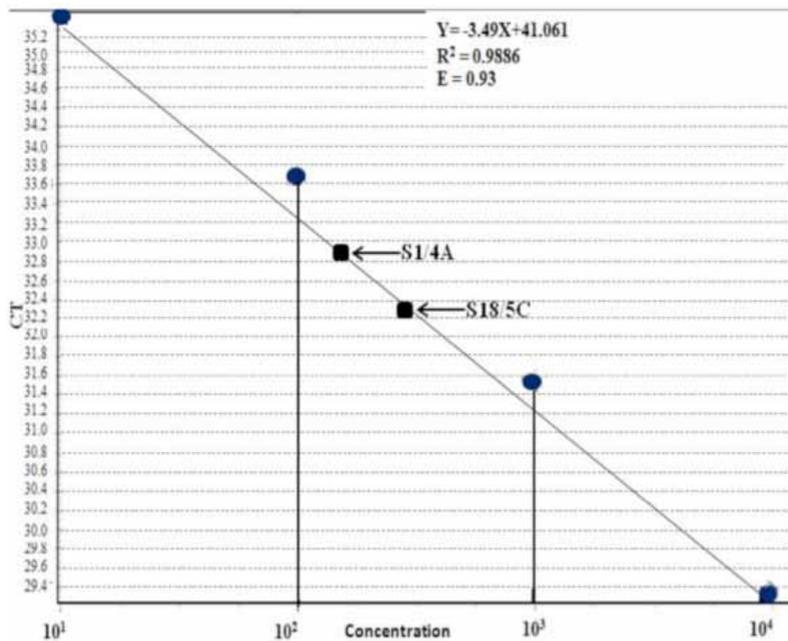


Fig 3 The standard curve of *T. gondii* positive control (●) at various concentrations from 10^1 to 10^4 and positive tested samples (■) at Ct = 32.28 and 32.91 located between 10^2 and 10^3 of the standard curve.

part of Thailand. Therefore, further studies with information based on farms and location of pigs will give a clearer understanding of distribution and mode of transmission of *T. gondii* in pigs.

Other methods have also been used to detect *T. gondii* contamination in pork meat, such as PCR-based assay targeting the *T. gondii* 529-bp repeat element and magnetic capture of *T. gondii* DNA [23-25]. However, these methods lack sensitivity, likely due to the non-homogeneous distribution of *T. gondii* tissue cysts, in combination with the small sample size. Nevertheless, Opsteegh *et al.* [24] found that the detection limit of their real-time PCR assay was approximately 230 tachyzoites per 100 g of meat sample. Therefore, they suggested the use of real-time PCR as an alternative method to bioassay for the detection and genotyping of *T. gondii*, and for quantifying the organism in meat samples from various sources.

This study found high numbers of contaminating *T. gondii*, at 21,900 and 33,100 bradyzoites per 100 g of minced pork. There were no information about the infective dose of *T. gondii* in humans, but Dubey [22] demonstrated

that, for rats, the infective dose by oral route was 10-1,000 bradyzoites. Our finding of heavy parasite loads in the pork meat samples far exceeded those levels, and therefore there is a need for urgent attention. If not properly cooked until the internal temperature is over 60°C [26], correctly smoked and cured in a brine solution of 2% sodium chloride, or frozen for 24 hours [25], pork meat will pose the high risk of *T. gondii* transmission. Health education should be given to the most vulnerable groups, such as pregnant women and immunocompromised individuals, and the general consumer group [1].

The seroprevalence of *T. gondii* in pigs is on a general downward trend, 0-64% in fattened pigs, and 3-31% in breeding females in Europe [6]. In the USA, the prevalence was as low as 0.9-28.8% due to the modern farm management systems including cat and rodent control [6]. Higher prevalence (60-68%) was found in some parts of China and Africa, with free-range animals that were not kept in enclosed and controlled spaces [3,6,24]. Apart from the husbandry management protocols, the detection methods used in large

animal studies may affect the results. *T. gondii* infection was detected by indirect methods, such as serological procedure or direct method by bioassay in mice, which require about 8 weeks until the results are available. The uncertainty of the sampling technique for the detection of *T. gondii* in large animals is another limiting factor. Highly sensitive and specific tools, such as real-time PCR assay, are preferable, particularly in low prevalence situations. Quantification is also necessary, because despite low contamination-levels, there may be high parasite loads, making pork an important source of toxoplasmosis.

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