Genotyping of *Coxiella burnetii* from ruminant placentas by Single-Nucleotide Polymorphism (SNP)

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Abstract

Coxiella burnetii (*C. burnetii*) is etiology of Q fever in ruminants and can be transmitted from animal to human as zoonotic pathogenic agent. Recently it has been identified as the cause of endocarditis in multiple cases in humans in northeast Thailand. However, genotyping of *C. burnetii* in animal is still unknown. We aimed to identify the subtype (ST) of *C. burnetii* in ruminants in the northeast, north, central and west of Thailand to develop benefit baseline data for Q fever molecular epidemiological study by Single-Nucleotide Polymorphism (SNP) analysis. We undertook a cross-sectional study, using 1,049 placentas of healthy ruminants as the sample, and real-time PCR as the diagnostic method for DNA detection. If the result show strong positive, which has low cycle thresholds (Ct), we proceeded with SNP analysis with a panel of 7 Taqman SNP assays and 6 melt analysis of Mismatch Amplification Mutation Assays (Melt-MAMA) for knowing subtype of the organism. Our results, 805 placenta samples, were positive by real time PCR. From those results, we chose 76 strong positive samples, which they were DNA of cow, buffalo, and goat placentas. The SNP results show that *C. burnetii* from placentas were 2 subtypes, which included 46.05% (35/76) of ST20 and 44.74% (34/76) of ST18/25. This provides useful information for molecular epidemiological study of *C. burnetii* that need further attention in the future for One Health collaboration.

Keywords: *Coxiella burnetii*, ruminant placentas, single-nucleotide polymorphism, Thailand

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การหา genotype ของเชื้อ *Coxiella burnetii* จากรกสัตว์เคี้ยวเอื้องด้วยวิธี Single-Nucleotide Polymorphism (SNP)

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บทคัดย่อ

Coxiella burnetii (C. burnetii) เป็นสาเหตุของโรคไข้คิวในสัตว์เคี้ยวเอื้องที่สามารถติดต่อระหว่างสัตว์สู่คน ในช่วงหลายปีมานี้มีรายงานผู้ป่วยมีอาการกล้ามเนื้อหัวใจอักเสบที่เกิดจากเชื้อนี้หลายรายในภาคตะวันออกเฉียงเหนือของประเทศไทย อย่างไรก็ตามยังไม่มีการศึกษาจำเพาะเรื่องการหา genotype ของเชื้อนี้ในสัตว์ ทีมผู้วิจัยจึงทำการตรวจหา subtype (ST) ของ *C. burnetii* จากรกสัตว์เคี้ยวเอื้องในภาคตะวันออกเฉียงเหนือ ภาคเหนือ ภาคกลางและภาคตะวันตกของประเทศไทยด้วยวิธี Single-Nucleotide Polymorphism (SNP) เพื่อให้ได้ข้อมูลพื้นฐานที่เป็นประโยชน์ต่อการศึกษาโรคไข้คิวทางระบาดวิทยาเชิงพันธุกรรมต่อไป โดยได้ทำการศึกษาแบบ cross-sectional โดยการเก็บตัวอย่างรกของสัตว์เคี้ยวเอื้องที่มีสุขภาพดีจำนวน 1,049 ตัวอย่าง นำมาตรวจหาสารพันธุกรรมด้วยวิธี real-time PCR และหากได้ผลบวกแรง โดยมีค่า cycle threshold ต่ำ จากนั้นตรวจวินิจฉัยต่อด้วยวิธี SNP โดยแบ่งเป็น 7 Taqman SNP assays และ 6 melt analysis of Mismatch Amplification Mutation Assays (Melt-MAMA) เพื่อหา subtype ของเชื้อนี้ ผลการศึกษาพบตัวอย่างรกให้ผลบวกด้วยวิธี real time PCR จำนวน 805 ตัวอย่าง จึงเลือกตัวอย่าง DNA ที่ให้ผลบวกแรง ซึ่งเป็น DNA ของรกโค กระบือ และแพะ รวมกันจำนวน 76 ตัวอย่าง และเมื่อศึกษาด้วยวิธี SNP พบว่าเชื้อ *C. burnetii* จากรกมี 2 subtype คือ ST20 46.05% (35/76) และ ST18/25 44.74% (34/76) ข้อมูลนี้มีประโยชน์ต่อการศึกษาทางระบาดวิทยาเชิงพันธุกรรมและเป็นข้อมูลที่น่าสนใจต่อไปสำหรับการศึกษาในแบบสุขภาพหนึ่งเดียว

คำสำคัญ: *Coxiella burnetii* รกสัตว์เคี้ยวเอื้อง single-nucleotide polymorphism ประเทศไทย

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Introduction

Q fever is a zoonotic disease caused by the gram-negative intracellular bacterium, *Coxiella burnetii* (*C. burnetii*). Many species of animals can act as hosts for *C. burnetii*, but the main reservoirs are ruminants. The transmission route is inhalation of the bacteria from dried animal waste products, with particularly high amounts in birth products; placenta, amniotic fluid, vaginal mucous. Infected placentas from humans and animals can contain large numbers of *C. burnetii* (Angelakis and Raoult, 2010). Human Q fever cases have been identified in Thailand, mainly in northeast region (Pachirat *et al*., 2012; Suputtamongkol *et al*., 2003; Watt *et al*., 2014), but the investigation of the source of Q fever in humans has been unrewarding. A few studies from animal and livestock sites in Thailand have been performed previously (Muramatsu *et al*., 2014; Yingst *et al*., 2013)**.**

The diagnostic methods which are available include serology tests, polymerase chain reaction (PCR), and immunohistochemistry (IHC) (Bielawska-Drozd et al., 2014; Christensen *et al*., 2006; OIE, 2016). Bacterial culture and isolation are not commonly used, because *C. burnetii*, a potential bioterrorism agent, must be processed in a biosafety level 3 laboratory. However, genotyping methods may occasionally be useful for molecular epidemiology without culture. Genotyping methods include multi loci variable number of tandem repeats analysis (MLVA) (Cumbassá *et al*., 2015; OIE, 2016), multispacer sequence typing (MST) (Bielawska-Drozd *et al*., 2014; OIE, 2016), whole genome PCR scanning (WGPS) (Sidi-Boumedine *et al*., 2015a), whole genome sequencing (WGS) (Walter *et al*., 2014), and single-nucleotide-polymorphism (SNP), using either Taqman or melt analysis of Mismatch Amplification Mutation Assays (melt-MAMA) (Hornstra *et al*., 2011; Karlsson *et al*., 2014; OIE, 2016). Some of these techniques are complicated to interpret, and some techniques tend to fail without highly purified bacterial DNA from isolation. Given sufficient DNA, the SNP method is rapid, cost-effective, and safer for lab technicians compared to bacterial culture/isolation, while still providing critical information (Karlsson *et al*., 2014; Pearson *et al*., 2014).

The subtypes (ST) of *C. burnetii* are often associated with host species, based on the studies in USA and European countries (Bauer *et al*., 2015; Galiero *et al*., 2016; Sidi-Boumedine *et al*., 2015a). It has been used to show links between Q fever outbreaks, for example, in the Netherlands (Huijsmans *et al*., 2011; Tilburg *et al*., 2012), whereas without genotyping information, definitive connections would not have been identified. However, there is no information of subtypes of *C. burnetii* in Thailand. We aimed to identify the subtypes of the *C. burnetii* from the positive ruminant placentas by SNP genotyping.

Materials and Methods

Study area

The cross-sectional study ran from August 2012 to September 2013. The study areas were mainly in the northeast region, which had human case reports. Some northeast people have the placenta consumption behavior, which might be a risk of infection. In addition, some people in the north region had the same behavior as the northeast people, so we expanded the studies areas to the north. The central and the west regions have high density of ruminants, justifying sample collection there as well.

Sample collection

The veterinary officers of the Department of Livestock Development collected the cotyledonary area of ruminant placenta from farms and fresh markets by convenience

sampling. Key information including the owner, species, age of the ruminants, gross appearance of the placenta, and parturition time were recorded in a one-page form. The placenta was kept frozen until sent on ice to the immunology section, National Institute of Animal Health (NIAH).

Laboratory investigation

The placenta samples were extracted for DNA by DNeasy blood and tissue kit (Qiagen, Germany), according to manufacturer's instructions. Extracted nucleic acid was tested by real-Time PCR targeting on IS1111 gene on a Roche Lightcycler 2.0 machine at NIAH (Christensen *et al*., 2006; Jones *et al*., 2011; Sidi-Boumedine *et al*., 2015b). The strong positive placenta samples which had low cycle threshold (Ct) in the range of 9.24 to 29.98, were chosen (Yingst *et al*., 2013). The samples were tested by SNP technique with ABI7500 fast machine real time PCR (Applied Biosystems, USA) platforms using SYBR Green DNA bind dye as a component of Platinum SYBR Green qPCR Supermix-UDG (Thermo Scientific, USA) at the virology laboratory, Armed Forces Research Institute of Medical Sciences (AFRIMS). The SNP assays included seven Taqman assays; 57bp327, 22bp91, 56bp10, 18bp376, 51bp356, 18bp166, 37bp215 (Pearson *et al*., 2014), and six Melt MAMA assays were 18bp34, 22bp118, 51bp67, 20bp155, 5bp109, 51bp492 (Hornstra *et al*., 2011). The positive results were interpreted by the fluorescence color matching at the single nucleotide base position for Taqman assay. The specific melting temperature (°C) of each assays were the positive results in Melt MAMA.

Data analysis

The results of subtype were identify by using *C. burnetii*'s SNP dichotomous tree (Figure 1) (Hornstra *et al*., 2011; Pearson *et al*., 2014; Pearson *et al*., 2013). The results were descriptive data.

Results

We collected 1,049 ruminant placentas. Eight hundreds placentas were positive by real-time PCR. From positive samples, the strong positive PCR placenta samples were 76 samples, which from 48 sources of placenta (44 farms and 4 fresh markets) (Table 1). The youngest animal was 2 years old and the oldest animal was 11 years old. The range of parturition time was once to seven times. Almost all of the placenta were normal grossly, and were not from abortion cases. The only exception being a retained placenta of a beef cow was in this study.

Table 1 Results of genotyping of *C. burnetii* DNA samples (N = 76) by single-nucleotide polymorphism technique, from 48 sources of placenta.

Species	Number of sample	Number of placenta		Number of positive subtype		
		Farm	Fresh market	ST20	ST18/25	Undetermined
Dairy cow	40	40	-	14	22	4
Beef cow	26	26	-	15	10	1
Buffalo	8	4	4	4	2	2
Dairy goat	1	1	-	1	-	-
Meat goat	1	1	-	1	-	-
Total	76	72	4	35	34	7

Base on SNP dichotomous tree, we found two subtypes (ST), which were ST20 and ST18/25 (Figure 1). ST20 was positive by 57bp327, 22bp91, 5bp109, and 56bp10 assays, whereas ST18/25 was positive by 57bp327, 22bp91, 18bp166, and negative by 51bp492, and 37bp215 assays. ST20 was found 46.05% (35/76), ST18/25 was found 44.74% (34/76), and undetermined subtype samples were 9.21% (7/76) (Table 1). Our main area of the study, the northeast and the north regions, had both ST20, and ST18/25 in dairy cows, beef cows, and buffaloes. In the west region, all subtyped samples were ST20. In the central region, we found ST18/25 (Figure 2). It seems that each subtype did not clearly separate by the region of Thailand.

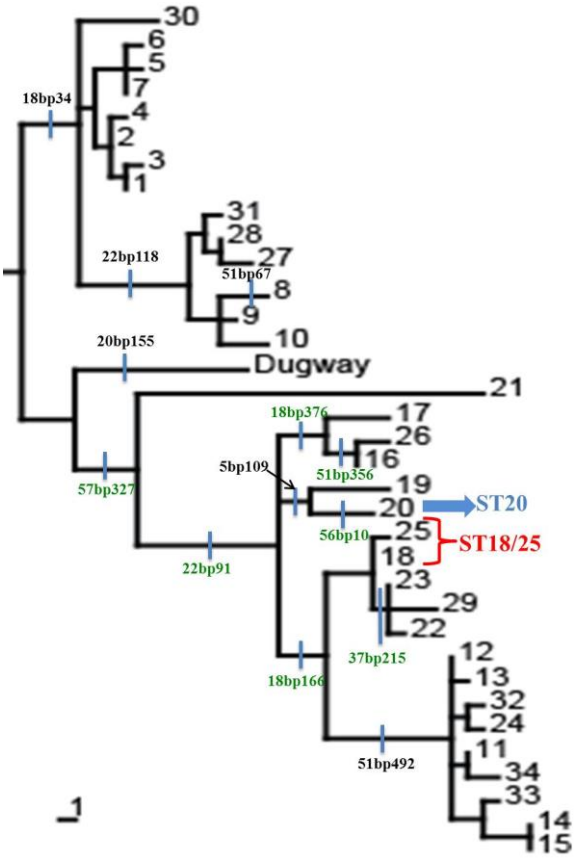

Figure 1 Phylogenetic tree of *C. burnetii* genotype (Hornstra *et al*., 2011; Pearson *et al*., 2014; Pearson *et al*., 2013). Taqman (7 green colored locus names) and Melt MAMA (6 black colored locus names) assays' results were interpreted by negative or positive to each target from the root of the tree (on the left of the figure) to the terminal nodes (on the right of the figure). Numbers at the end of terminal nodes indicated the subtypes of *C. burnetii*. Colored arrows were subtypes that found in ruminants in Thailand: blue arrow was ST20 and red arrow was ST18/25.

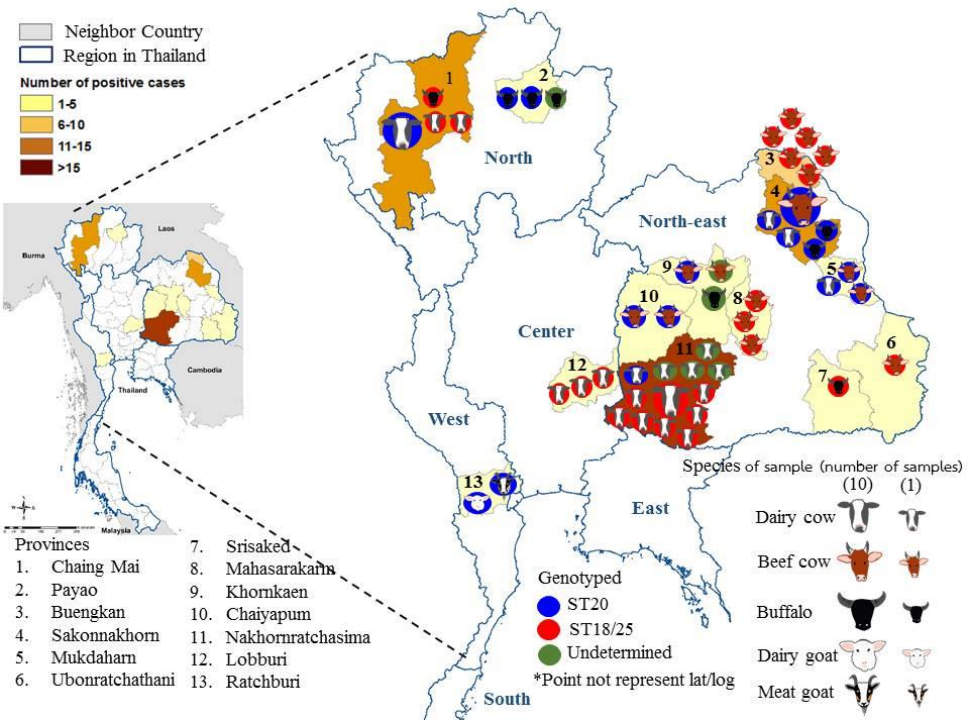

Figure 2 Map shows the 13 provinces of Thailand where the placenta samples were collected from August 2012 to September 2013.

Discussion

The cow and buffalo PCR positive placentas were roughly equally ST20 positive and ST18/25 positive. According to the results of ST18/25, we could not distinctly identify the ST18 and ST25 from each other, because our positive DNA amount might not enough to be differentiated by the assays. The goat positive placentas (though very few in number) were both ST20 (Table 1). Our study found ST20 in both dairy and meat purposed animals. These results of ST20 in meat purposed animal, which including a beef cow with retained placenta, were additional evidence of ST20 in animals to other studies such as in the US, France, Hungary, Italy, Germany, the Netherlands, and Spain, which ST20 was commonly found in dairy products, milk, and placenta (Astobiza *et al*., 2012; Bauer *et al*., 2015; Di Domenico *et al*., 2014; Pearson *et al*., 2014; Sidi-Boumedine *et al*., 2015a; Tilburg *et al*., 2012). ST20 was also found in clinical human samples in France (Astobiza *et al*., 2012). It was interesting that ST20 was found in human and therefore we cannot neglect to the disease even there was no abortion case in our study. However, the limitation of our final summary was we did not have ST information in human cases in Thailand. Our results provide data for the future surveillance and it is useful for Q fever investigation in the future. The additional analyses of samples from both human and animals cases are needed to complete the questions of *C. burnetii* infection for one health approach.

Conclusion

As results from this study, it showed 2 subtypes of *C. burnetii* in ruminants in Thailand. The subtypes provide useful information for more molecular epidemiological studies of *C. burnetii* that need further attention in the future for One Health collaboration. Due to the lack of ST information in human cases in Thailand, we cannot show any molecular epidemiological link between any prospective human cases and a possible animal source.

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