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A nationwide survey of pathogenic leptospires in urine of cattle and buffaloes by Loop-mediated isothermal amplification (LAMP) method in Thailand, 2011–2013

Duangjai SUWANCHAROEN[1)], Supaluck LIMLERTVATEE[2)], Philaiphon CHETIYAWAN[2)], Phichet TONGPAN[2)], Nongluck SANGKAEW[2)], Yaowarat SAWADDEE[2)], Kanya INTHAKAN[2)] and Anuwat WIRATSUDAKUL[3)]*

[1)]*National Institute of Animal Health, Department of Livestock Development, 50/2 Kasetklang, Ladyao, Chatuchak, Bangkok 10900, Thailand*

[2)]*Regional Veterinary Research and Development Center, Department of Livestock Development, 50/2 Kasetklang, Ladyao, Chatuchak, Bangkok 10900, Thailand*

[3)]*Department of Clinical Sciences and Public Health, and the Monitoring and Surveillance Center for Zoonotic Diseases in Wildlife and Exotic Animals, Faculty of Veterinary Science, Mahidol University, 999 Phuttamonthon Sai4 Rd., Salaya, Phuttamonthon, Nakhon Pathom 73170, Thailand*

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ABSTRACT. Leptospirosis is a worldwide distributed zoonosis which has long been endemic in Thailand. Cattle and buffaloes are important livestock species that live in close contact with humans, especially in rural areas. These animals may, therefore, act as long-term carriers of leptospirosis for humans and other livestock species. The present study employed loop-mediated isothermal amplification (LAMP) method to detect pathogenic leptospiral 16S rDNA in the urine of cattle and buffaloes for assessing associations between uroprevalence and species, sex, age and spatial distribution. A total of 3,657 urine samples were collected for laboratory diagnosis, and 312 of which turned positive to the test (true prevalence 5.90%; 95% CI 4.98–6.91). The highest true uroprevalence was found in lower northern region at 19.80% (95% CI 15.83–24.32) followed by upper and lower northeastern regions at 15.22% and 6.25%, respectively. However, the highest true uroprevalence in beef cattle, the majority of cattle in Thailand, was recorded in northeastern region which is the endemic area of human leptospirosis. The uroprevalence was not statistically different among species and types of examined animals. Male animals were over twice more likely to be infected compared to females. Excluding animals younger than one year of age due to small sample size, the uroprevalence upraised with increasing age. A collaborative investigation between veterinary and public health sectors is required to holistically explore the link between leptospirosis in humans and livestock, especially in high prevalent areas.

KEY WORDS: epidemiology, LAMP, leptospirosis, livestock, urine

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Leptospirosis is one of the major bacterial zoonotic diseases worldwide. Recently, the WHO Leptospirosis Burden Epidemiology Reference Group (LERG) estimated number of global severe human leptospirosis cases to over 500,000 per year [1]. However, this number seems to be underestimated due to inadequate surveillance and difficulties in disease diagnosis. The etiological agent responsible for the disease is pathogenic Gram-negative bacteria of the genus *Leptospira* [16]. Indeed, bacteria in this genus are divided into pathogenic and nonpathogenic species. More than 250 serovars of pathogenic *Leptospira* spp. were now discovered and further clustered into 24 serogroups [6].

Leptospires colonize the proximal renal tubules of carrier and maintenance hosts, and the bacteria are intermittently excreted in the urine. Humans and animals are mainly infected by exposure to contaminated water or soil or by direct contact with infected animals [2]. Persistent infection of the reproductive tract dominantly manifests in ruminant cases, especially when serovars Hardjo is involved. In cattle, leptospirosis has been accounted as a major cause of reproductive failures, such as abortions, still-birth and weak off-spring, that consequently lead to unquantified economic loss to the farmers [13].

In Thailand, human leptospirosis was firstly recognized in 1942 and became one of the 58 reportable infectious diseases under the national passive surveillance system in 1972 [23]. Since then, the disease has been continuously reported. Morbidity rate of the disease was as high as 23.13 per 100,000 populations with mortality rate of 0.59 per 100,000 populations. In the last decade, the annual morbidity rate was in the range of 4.83 to 8.57 per 100,000 populations [4]. The disease has been found mostly in the Northeastern region of Thailand, and the main affected occupation is rice farmer.

Cattle and buffaloes have been domesticated and used in agricultural countries like Thailand since ancient time. Formerly, they were used as a tool in rice cultivation. With the development of agricultural technology, cattle and buffaloes were replaced with machinery tools. However, Thais, espe-

*CORRESPONDENCE TO: WIRATSUDAKUL, A., Department of Clinical Sciences and Public Health, and the Monitoring and Surveillance Center for Zoonotic Diseases in Wildlife and Exotic Animals, Faculty of Veterinary Science, Mahidol University, 999 Phuttamonthon Sai4 Rd., Salaya, Phuttamonthon, Nakhon Pathom 73170, Thailand.

e-mail: anuwat.wir@mahidol.edu, neoart23026@gmail.com

Table 1. Standardized uroprevalence (%) of pathogenic leptospires found in urine of cattle and buffaloes tested with LAMP and prevalence ratio using beef cattle as a reference

Species and type of animals	*N*[a)]	*n* + *ve*[b)]	Standardized uroprevalence (%) (95% CI)[c)]	Prevalence ratio (95% CI)
Beef cattle	2,142	171	5.31 (4.17–6.62)	Reference
Dairy cattle	1,027	95	6.66 (4.93–8.72)	1.24 (0.93–1.66)
Beef buffalo	488	46	6.85 (4.42–9.96)	1.27 (0.87–1.85)
Total	3,657	312	5.90 (4.98–6.91)	1.11 (0.89–1.38)

Remarks: a) *N*=number of urine samples tested. b) *n* +*ve*=number of positive samples. c) 95% CI=95% confidence interval.

cially who live in rural areas, still raise them, but change the purpose to mainly produce for supplying the food market. According to the records of Department of Livestock Development (DLD) of Thailand in 2014, 4.31 million beef cattle were raised by 7.45 hundred thousand households across the country, while numbers of dairy cattle and buffaloes were 0.51 and 0.84 million which were raised in 0.16 and 1.85 hundred thousand households, respectively [7]. Interestingly, 50% of these animals were raised in Northeastern region which is the most prevalent area of human leptospirosis in Thailand [4]. In rural agricultural communities, cattle and buffaloes live in close contact with humans, and these animals are potential to be a major reservoir for human infections [8, 10].

Leptospirosis is basically diagnosed by dark-field microscopy or culture which has low sensitivity and is time consuming. To increase sensitivity, molecular techniques like PCR have been employed [21]. However, PCR is still time consuming and requires sophisticated machines and skillful interpretation. To solve this problem, another molecular technique called loop-mediated isothermal amplification (LAMP) was developed. This new technique allows us to simply and rapidly diagnose the pathogen with high specificity. Unlike PCR, the amplification of a target DNA sequence under isothermal conditions in LAMP is achieved in approximately 1 hr, and the amplified products can be easily observed with the naked eyes [15]. Thus, LAMP is highly applicable in resource-limited country like Thailand.

The aims of the present study were to determine the occurrence of pathogenic leptospires in populations of cattle and buffaloes in the national scale of Thailand using LAMP as a diagnostic method and to assess associations between uropositivity and species, sex, age and spatial distribution.

MATERIALS AND METHODS

Study design: To investigate the prevalence of pathogenic leptospires infection in cattle and buffaloes throughout Thailand, a cross-sectional study was conducted in all nine livestock administrative regions, as delineated by the DLD, including the central, eastern, western, upper and lower northern, upper and lower northeastern, and upper and lower southern regions. Three provinces in each region were randomly selected. A total of 27 provinces were chosen in this study. The sampling frame in each province was prepared by provincial DLD livestock officers who own the animal population data.

Sample collection and laboratory examination: Ten m*l* of urine were collected from each animal by field veterinarians during January 2011 and February 2013 and submitted to National Institute of Animal Health in Bangkok and Regional Veterinary Research and Development Center located in each livestock administrative region for laboratory diagnosis. The samples were kept in cool storage (4°C) for further processing. The urine was then centrifuged at 10,000 g for 10 min and washed out twice with 1 m*l* of phosphate buffered saline to obtain pellets. The pellets were subsequently examined for the presence of pathogenic leptospiral 16S rDNA by LAMP. The LAMP technique in this study was performed following the instruction described in a previous study [21]. In this technique, the reaction mixture was amplified at 61°C for 90–120 min in a dry bath incubator (Major Science, New Taipei City, Taiwan) and subsequently heated at 80°C at the end of the process. The samples with the presence of fluorescence (green color), as observed by eyes, were classified as positive.

Data analysis: The associations of uroprevalence and species, age and sex were analyzed in the present study. True prevalence and prevalence ratio were calculated with functions provided in the package '*epiR*' of statistical programming language R version 3.2.2 (R Development Core Team, Vienna, Austria). Sensitivity and specificity of the LAMP, which were estimated at 96.8% and 97.0% [22], were taken into account in the calculation of true prevalence as required in the algorithms.

RESULTS

A total of 3,657 urine samples were collected from 2,142 beef cattle, 1,027 dairy cattle and 488 beef buffaloes, and 312 of which turned positive to the test. Overall true uroprevalence was 5.90% (95% CI 4.98–6.91). The highest uroprevalence was found in beef buffaloes at 6.85% (95% CI 4.42–9.96) followed by dairy cattle and beef cattle at 6.66% (95% CI 4.93–8.72) and 5.31% (95% CI 4.17–6.62), respectively (Table 1). However, the difference of the uroprevalence among these animals was not statistically significant.

The standardized uroprevalence and prevalence ratio for each livestock administrative region are shown in Table 2. The highest uroprevalence was found in lower northern region at 19.80% (95% CI 15.83–24.32) followed by upper and lower northeastern regions at 15.22% (95% CI 11.4–19.8)

Table 2. Standardized uroprevalence (%) of pathogenic leptospires found in urine of cattle and buffaloes tested with LAMP in each livestock administrative region of Thailand and prevalence ratio using lower southern region as a reference

Region	N[a)]	$n + ve$[b)]	Standardized uroprevalence (%) (95% CI)[c)]	Prevalence ratio (95% CI)
Central	460	32	4.22 (2.10–7.10)	2.78 (1.12–6.90)
East	412	21	2.24 (0.38–4.97)	1.47 (0.53–4.09)
Lower north	408	88	19.80 (15.83–24.32)	13.37 (5.90–30.28)
Upper north	431	20	1.75 (0.03–4.33)	1.25 (0.44–3.57)
Lower northeast	406	36	6.25 (3.70–9.63)	4.15 (1.72–10.00)
Upper northeast	353	61	15.22 (11.4–19.8)	10.30 (4.49–23.65)
Lower south	404	18	1.55 (0.00–4.19)	Reference
Upper south	383	31	5.43 (2.94–8.81)	3.69 (1.51–9.05)
West	400	5	0.00 (0.00–0.00)	0.00 (0.00–0.00)

Remarks: a) *N*=number of urine samples tested. b) *n +ve*=number of positive samples c) 95% CI=95% confidence interval.

Table 3. Number of urine samples (*N*) tested with LAMP for the presence of pathogenic leptospires in each species and type of animals and number of positives (*n +ve*), with percent of true prevalence in parentheses, in each livestock administrative region of Thailand

Region	Species and types of animals					
	Beef cattle		Dairy cattle		Beef buffaloes	
	N	*n + ve* (%)	*N*	*n + ve* (%)	*N*	*n + ve* (%)
Central	251	1 (0.00)	209	31 (12.61)	0	0 (0.00)
East	150	4 (0.00)	171	14 (5.53)	91	3 (0.32)
Lower north	166	16 (7.08)	137	44 (31.04)	105	28 (25.23)
Upper north	245	15 (3.33)	137	1 (0.00)	49	4 (5.50)
Lower northeast	130	34 (24.68)	183	2 (0.00)	93	0 (0.00)
Upper northeast	238	58 (22.78)	0	0 (0.00)	115	3 (0.00)
Lower south	380	18 (1.85)	24	0 (0.00)	0	0 (0.00)
Upper south	252	20 (5.26)	96	3 (0.13)	35	8 (21.17)
West	330	5 (0.00)	70	0 (0.00)	0	0 (0.00)

and 6.25% (95% CI 3.70–9.63), respectively. Focusing on prevalence ratio, the uroprevalence in lower northern and upper northeastern regions was 13.37 (95% CI 5.90–30.28) and 10.30 (95% CI 4.49–23.65) times, respectively, compared to the lower southern reference region.

Table 3 demonstrates number of animals tested and number of positives collected from each livestock administrative region as observed in each species and types of animals. The highest uroprevalence in beef cattle was found in lower northeastern region at 24.68%, whereas the highest uroprevalence in dairy cattle and beef buffaloes was recorded in lower northern region at 31.04% and 25.23%, respectively. The overall true uroprevalence and the prevalence in each species are illustrated by regions in Fig. 1.

Associations between uroprevalence and sex as well as age were considered together in cattle and buffaloes and expressed as prevalence ratios for four age groups and also separately for sex as illustrated in Table 4. Female and age between one and five years were used as reference categories for sex and age, respectively. Males were 2.85 (95% CI 2.07–3.94) times more likely to be uropositive than females. Excluding animals aged younger than one year due to small sample size, uropositivity trended to increase with increasing age.

DISCUSSION

The present study provides baseline information on the uroprevalence of the pathogenic leptospires in the population of cattle and buffaloes in Thailand during 2011–2013. The method used in this study, which is LAMP, has been more and more employed for detection of various types of pathogens due to its simplicity and less sophisticated machinery approaches. LAMP has been applied for detection of 18 World Organization for Animal Health (OIE) notifiable viral diseases of ruminants, swine and poultry as reviewed in a previous study [14]. In bacterial diseases, LAMP has also been recently used for a rapid and reliable diagnosis of many bacterial pathogens, for examples, *Brucella abortus* [11], *Campylobacter jejuni* [18], *Coxiella burnetii* [19] and *Salmonella enterica* serovar Typhi [9]. Moreover, the LAMP applied in the detection of pathogenic leptospires in this study was previously reported to detect as low as 10–100 copies of rDNA [21]. Hence, this method makes it possible

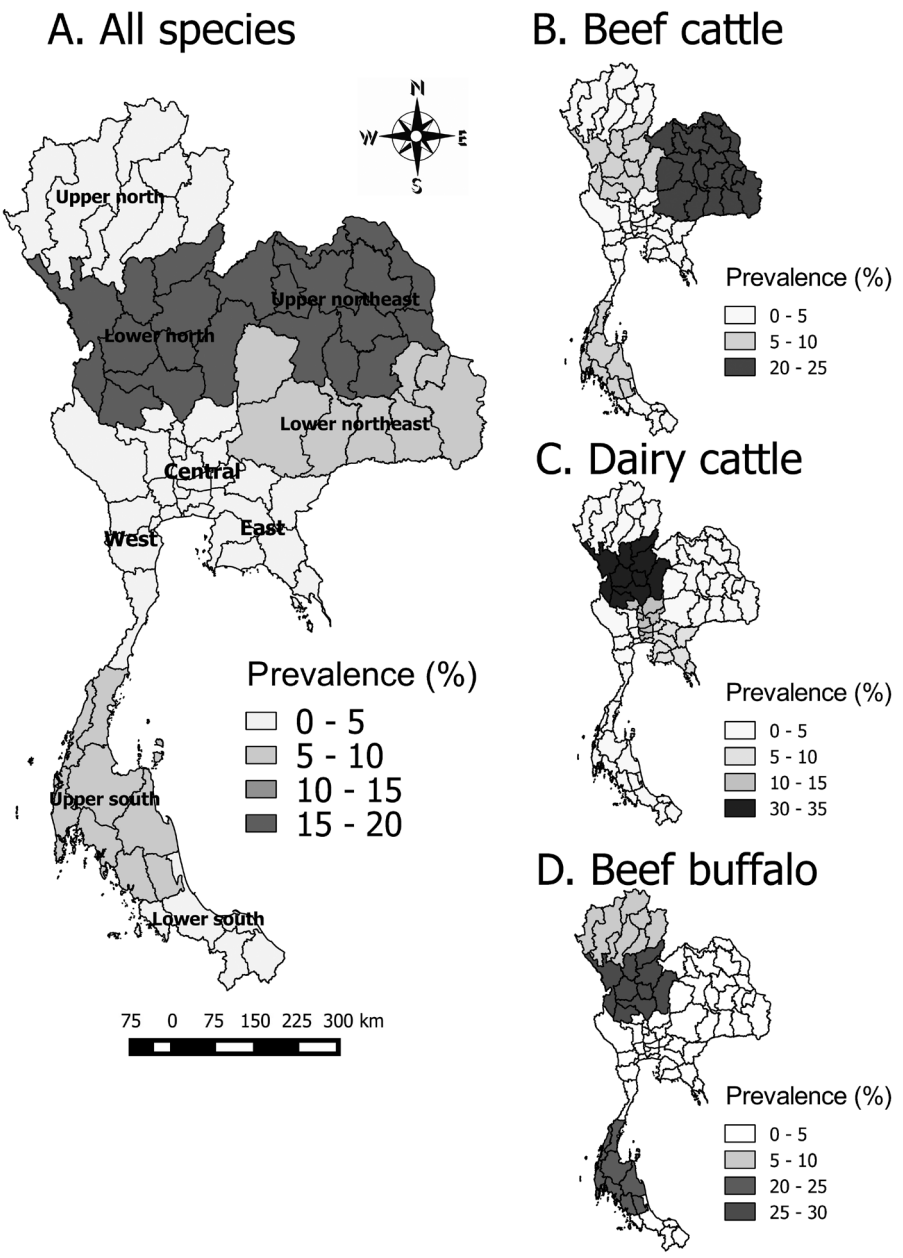

Fig. 1. True prevalence (%) of animals tested with LAMP for the presence of pathogenic leptospires in all and each species (A. All species, B. Beef cattle, C. Dairy Cattle and D. Beef buffalo) in all nine livestock administrative regions in Thailand.

to detect even low number of genetic materials. Moreover, the simplicity of LAMP allowed us to train regional laboratory staff to conduct the tests themselves. It was not necessary any more to submit the samples from remote provinces to the central laboratory in Bangkok. Thus, the samples were obviously transported in a shorter distance and time, resulting in a better chance to detect the pathogens. However, this technique does not provide any genotypic information, such as strains of the pathogens. Thus, it is not possible to compare the strains of pathogenic leptospires in this study.

In the present study, the uroprevalence of pathogenic leptospires was not significantly different among cattle and buffaloes. Our result was in line with the previous study [3]. Although the beef cattle and beef buffaloes are free-ranging animals that can easily contact with the pathogens in the fields and dairy cattle are mainly raised in houses, dairy cattle are possible to be infected by contacting with urine of rats and other infected cattle within the houses as rats were indicated as an important source of infection in dairy cattle farms [17]. The epidemiology of leptospirosis transmission

Table 4. Standardized uroprevalence (%) of pathogenic leptospires found in urine of cattle and buffaloes tested with LAMP as distinguished by sex and age of animals and prevalence ratio using female and age at 1–5 years as references, respectively

Sex/Age	N[a)]	$n + ve$[b)]	Standardized uroprevalence (%) (95% CI)[c)]	Prevalence ratio (95% CI)
Sex				
Female	1,928	187	7.14 (5.82–8.64)	Reference
Male	191	42	20.24 (14.61–27.06)	2.85 (2.07–3.94)
No data	1,538	83	2.56 (1.47–3.88)	0.35 (0.25–0.50)
Age				
<1 year	11	2	16.19 (2.28–47.65)	2.79 (0.78–9.98)
1–5 years	1,028	94	6.55 (4.83–8.59)	Reference
6–10 years	347	55	13.70 (10.00–18.19)	2.12 (1.50–3.01)
>10 years	62	14	20.87 (11.68–33.48)	3.22 (1.88–5.50)
No data	2,209	147	3.90 (2.87–5.09)	0.60 (0.44–0.81)

Remarks: a) N=number of urine samples tested. b) $n + ve$=number of positive samples. c) 95% CI=95% confidence interval.

patterns in dairy cattle rearing system in Thailand should be further investigated.

Spatially, the highest uroprevalence was observed in lower northern region instead of northeastern region which is the endemic area of leptospirosis in humans [4]. The most likely explanation is that lower northern region is one of the main cattle and buffalo trading areas. Thus, the highly dynamic movements of animals may facilitate the spread of pathogenic leptospires among cattle and buffaloes in this region. However, a further investigation shown in Fig. 1 and Table 3, which separately analyzed uropositivity in each region by species and types of animals, reveals that uroprevalence in beef cattle in both lower and upper northeastern regions was apparently higher than other remaining regions. This finding was in accordance with a previous study on a nationwide survey of leptospirosis in Thailand performed in 2001 [20]. As the vast majority of cattle in Thailand are beef cattle (89.4%), northeastern region should still be considered the main endemic area for leptospirosis in cattle in this country. This particular region has also long been endemic area of leptospirosis in humans. More comprehensive investigations on association between leptospirosis in livestock and humans should be seriously initiated in the region. Even though beef cattle and beef buffaloes are both raised in free-ranging system, it was noticeable that the uroprevalence in beef cattle was much higher than that of beef buffaloes in northeastern region but it was opposite in the lower north. This inconsistency was still poorly understood. A deeper investigation in the level of province or district instead of region is suggested to explain this phenomenon.

Regarding sex of animals, the risk of pathogenic leptospires infection was over twice in males compared to females. In general, natural breeding services have still been widely practiced in cattle and buffalo farming, especially in rural settings. This breeding system was previously identified as a risk factor for leptospirosis transmission [5]. As one male normally copulates with several females, the risk of infection is, therefore, higher in males. Artificial insemination with routine checking for the contamination of pathogenic leptospires should be introduced to the farmer in order to prevent the propagation of the disease by this route. The uroprevalence of pathogenic leptospires in cattle and buffaloes in this study increased with age of animals. This result was in agreement with a previous study conducted in Thailand [20]. However, our analysis on sex and age of animals should be interpreted with cautions due to high proportion of unrecorded data in these variables. The most possible explanation on the association of age and uroprevalence is that the increasing age of animals results in greater risk of exposure to the pathogens as older animals live longer compared to younger animals. Once animals expose to pathogenic leptospires and get infected, the animals may become chronic carriers and may shed the pathogen into environment for months or even years [12], resulting in a long-term source of infections for humans, cattle and other livestock species in the surrounding areas.

Nonetheless, this study is a cross-sectional one which provides a snap shot picture of leptospirosis infection in cattle and buffaloes in Thailand. A longitudinal study is strongly suggested in the future. This type of study may provide a better understanding on the risk factors and seasonal patterns which is beneficial for disease prevention and control. In this study, data recording practices during the field works make our epidemiological analysis less effective as we obtained a large proportion of unrecorded data on sex and age of animals. The problem, occurred, because the study was in a national scale, and therefore, there were many people in different parts of the country involved in the project. A better communication with local field staff and a better data management with consecutive data monitoring would be helpful in maintaining the obtained data in a good order. The improvement on this point would allow us to better explain the association between the observed uroprevalence and study factors. Moreover, the association between leptospirosis in livestock and humans was not investigated in the present study due to limited data on both sides. A collaborated research work among veterinary and medical researchers is proposed to overcome this limitation. The newly generated

data from this collaboration may provide us more comprehensive understanding on the links between leptospirosis in humans and animals especially livestock and make it possible to effectively prevent and control the disease.

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