Survey of Leptospirosis in free-grazing ducks in Thailand

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Abstract

Background: Free-grazing ducks have a natural feeding pattern that pose a risk of getting leptospires from environment. This study aims to investigate *Leptospira* spp. infection in free-grazing ducks from six provinces in lower northern Thailand where is a highly-populated area of free-grazing ducks. Even since past, study of poultry leptospirosis has not been addressed in Thailand.

Method: Three hundred and eight blood samples were collected. Sera were collected and tested for specific antibodies against 24 serovars of *Leptospira* spp. by using the microscopic agglutination test (MAT). Ten kidneys and fifteen cloacal swabs (five ducks/sample), collected from the flock with the highest titer, were subjected to the isolation and real time PCR targeting the *lipL32* gene, which specific to pathogenic *Leptospira* spp.

Result: MAT result shows overall seropositivity against *Leptospira* spp. at 88.3% and at least one serovar was found in each sample. The mostly detected serovars were Ranarum and Shermani at 87% and 77%, respectively. For real-time PCR results, the cloacal swabs and the kidney were positive to *LipL32* gene at 47% and 10%. However, *Leptospira* spp. could not be isolated because of bacterial contamination.

Conclusion: Based on MAT, the seroprevalence of Leptospirosis found in this study was relatively high in free-grazing ducks and genetic materials of pathogenic *Leptospira* spp. were found by real-time PCR. They might be one of the important reservoirs for disease transmission.

Keywords: Leptospirosis, *Leptospira* serovars, free-grazing ducks, lower northern Thailand

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การสำรวจโรคเลปโตสไปโรสิสในเป็ดไล่ทุ่งในประเทศไทย

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บทคัดย่อ

ที่มาของการศึกษา: เป็ดไล่ทุ่งมีรูปแบบการเลี้ยงปล่อยตามธรรมชาติจึงมีโอกาสติดเชื้อเลปโตสไปราจากสิ่งแวดล้อม การศึกษานี้เป็นการสำรวจการติดเชื้อเลปโตสไปราในเป็ดไล่ทุ่ง 6 จังหวัดในพื้นที่ภาคเหนือตอนล่างของประเทศไทย ซึ่งเป็นพื้นที่ที่มีการเลี้ยงเป็ดไล่ทุ่งมากพื้นที่หนึ่ง และที่ผ่านมายังไม่มีการศึกษาโรคเลปโตสไปโรสิสในสัตว์ปีกของประเทศไทย

วิธีการ: เก็บตัวอย่างเลือดเป็ดไล่ทุ่ง จำนวน 308 ตัวอย่าง ปั่นแยกซีรัมและนำมาทดสอบหาแอนติบอดีที่จำเพาะต่อเชื้อเลปโตสไปรา 24 ซีโรวาร์ ด้วยเทคนิค microscopic agglutination test (MAT) จากนั้น เก็บตัวอย่างไต จำนวน 10 ตัวอย่าง และ cloacal swab จำนวน 15 ตัวอย่าง (เป็ดไล่ทุ่ง 5 ตัวต่อ 1 ตัวอย่าง) จากฝูงที่ให้ผลบวกสูงสุดต่อการทดสอบด้วยวิธี MAT แล้วนำมาเพาะแยกเชื้อเลปโตสไปราในอาหารเลี้ยงเชื้อ และตรวจหายีน lipL32 ซึ่งมีความจำเพาะต่อเชื้อเลปโตสไปราชนิดก่อโรคด้วยเทคนิค real-time PCR

ผล: พบผลบวกของระดับภูมิคุ้มกันต่อเชื้อเลปโตสไปราจากการทดสอบด้วยวิธี MAT ร้อยละ 88.3 และพบอย่างน้อย 1 ซีโรวาร์ในทุกตัวอย่าง ซีโรวาร์ที่พบมากที่สุดคือ ซีโรวาร์ Ranarum และ Shermani ซึ่งพบร้อยละ 87 และ 77 ตามลำดับ ผลการทดสอบ real-time PCR พบตัวอย่าง cloacal swab และไต ให้ผลบวกต่อยีน LipL32 ร้อยละ 47 และ 10 ตามลำดับ แต่ไม่สามารถเพาะแยกเชื้อได้ เนื่องจากเกิดการปนเปื้อนของเชื้อแบคทีเรีย

สรุป: ระดับภูมิคุ้มกันต่อเชื้อเลปโตสไปราที่ตรวจพบด้วยวิธี MAT และการตรวจพบสารพันธุกรรมของเชื้อเลปโตสไปราชนิดก่อโรคโดยวิธี real-time PCR จากตัวอย่างเป็ดไล่ทุ่งในการศึกษานี้ สามารถใช้เป็นข้อมูลที่บอกว่าเป็ดไล่ทุ่งอาจเป็นสัตว์รังโรคที่สำคัญในการแพร่กระจายโรคเลปโตสไปโรสิส

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Introduction

Leptospirosis is a zoonotic disease affecting both warm and cold blooded vertebrates. It prevails throughout the world with high incidence in tropical and subtropical regions including Thailand. Due to the reduction in milk yield, abortion and infertility in mammals, leptospirosis is an economically important disease especially in livestock industry (Lilenbaum et al., 2014). Domestic animals may act as reservoir of leptospires and excrete through their urine. Transmission is via direct and indirect contact with the urine and contaminated environment.

In several countries, Leptospirosis has been reported in avian species, for example, the seropositivity of chickens were detected at 11% from Grenada and 11% from Trinidad by microscopic agglutination test (MAT) (Everard et al., 1985). Kidney samples from wild bird species in northern Botswana that were screened for the presence of *Leptospira* spp. showed 27.8% positive (Jobbins & Alexander, 2015). Furthermore, the study in poultry infected with *L. canicola* demonstrated death in chicken and duck embryos. In addition, the recovered animals showed symptoms and leptospiremia (Gleiser et al., 1955). Infected animals can shed leptospires to the environment especially water sources. Thus, water birds living in that area such as gulls and geese as well as swans and ducks can be infected to leptospires (Benskin et al., 2009; ECDC, 2006).

Leptospires contamination in water or soil is a major factor in numerous outbreaks and plays an important role in endemic area (Wynwood, 2014). Schneider et al. (2018) found pathogenic *Leptospira* spp. in 31% soil samples from Salvador, Brazil. Sehgal (2006) found *Leptospira* spp. at 1.9% in water from urban areas of Sarawak, Malaysia. In addition, a study revealed higher possibility of the pathogen presence in still water compared to rainwater and groundwater (Ganoza et al, 2006). Free-grazing ducks, most of them are laying ducks, have a natural feeding habit which is seeking rice seed falling and snails in rice paddies. Several pathogens can be transmitted to ducks by direct contact because of open free-range system (Songserm et al., 2006).

A previous survey of pathogenic leptospires in cattles and buffaloes disclosed that the true uroprevalence observed in the lower northern region of Thailand was the highest (Suwancharoen et al, 2016). And with the farming system as mentioned above, free-grazing ducks are at risk for the disease and could possibly be reservoir hosts. This study aims to detect the Leptospiral infection in free-grazing ducks in lower Northern Thailand as a preliminary survey for monitoring and control.

Materials and Methods

Study design

To investigate the prevalence of pathogenic leptospires infection in free-grazing ducks, samples were collected from six provinces in the lower northern Thailand including Kamphaengphet, Nakhon-Sawan, Phichit, Phitsanulok, Sukhothai and Uthaithani in 2017. Ten serum samples per flock from each district (50 samples per province except 58 samples from Kamphaeng Phet) were randomly collected. After MAT test, 10 kidneys and 15 pooled cloacal swabs (75 ducks total, 5 ducks per pool) were collected from the flock with the highest titer of leptospiral seropositivity.

Laboratory examination

Microscopic agglutination test

Serum samples were tested by MAT following the method as illustrated by Smythe (2008) at Leptospirosis Center, National Institute of Animal Health using 24 serovars of live *Leptospira* spp. antigen (Table 1). MAT titer of 1:50 and over was considered seropositivity.

Table 1. Twenty-four serovars of live *Leptospira* spp. antigen

Serogroups	Serovars	Strains
Australis	Bratislava (AUS)	Jez Bratislava
Autumnalis	Autumnalis (AUT)	Akiyami A
Ballum	Ballum (BAL)	Mus 127
Bataviae	Bataviae (BAT)	Swart
Canicola	Canicola (CAN)	HondUterecht IV
Celledoni	Celledoni (CEL)	Celledoni
Cynopteri	Cynopteri (CYN)	3522 C
Djasiman	Djasiman (DJA)	Djasiman
Grippotyphosa	Grippotyphosa (GRI)	Moskva V
Hebdomadis	Hebdomadis (HEB)	Hebdomadis
Icterohaemorrhagiae	Icterohaemorrhagiae (ICT)	RGA
Javanica	Javanica (JAV)	VeldratBataviae 46
Louisiana	Louisiana (LOU)	LSU 1945
Manhao	Manhao (MAN)	Li 130
Mini	Mini (MIN)	Sari
Panama	Panama (PAN)	CZ 214
Pomona	Pomona (POM)	Pomona
Pyrogenes	Pyrogenes (PYR)	Salinem
Ranarum	Ranarum (RAN)	ICF
Sarmin	Sarmin (SAR)	Sarmin
Sejroe	Sejroe (SEJ)	M 84
Shermani	Shermani (SHE)	1342 K
Tarassovi	Tarassovi (TAR)	Perepelitsin
Semaranga	Patoc (PAT)	Patoc I

Molecular detection

To confirm existence of *Leptospira* spp. genetic material, pieces of 1 cm^3 kidney and cloacal swabs which were collected in 9 ml of 1% bovine serum albumin supplemented with STAFF (sulfamethoxazole, 400 µg/ml; trimethoprim, 200 µg/ml; amphotericin B, 50 µg/ml; fosfomycin, 4 mg/ml; 5-fluorouracil, 1 mg/ml) at room temperature were tested by real-time PCR targeting *lipL32* according to Suwancharoen (2016B). The cycle thershold (Ct) value lower 40 was considered positive.

Isolation of Leptospira spp.

Samples were processed for bacterial culture in EMJH (Ellinghausen, McCullough, Johnson, Harris) selective media (Difco™, USA) with STAFF. All inoculated media were incubated aerobically at 30°C and examined under a dark-field microscope for the presence of *Leptospira* spp. once a week for 3 months (Benacer, 2013; OIE, 2014). Leptospires were considered positive by the characteristic of thin helical structure with prominent hooked ends and motility.

Data analysis

Seropositivity was calculated by number of positives divided by number of testing samples. True seropositivity was calculated following the formula where $prev_{adj}$, $prev_{obs}$, S_e, and S_p stand for adjusted prevalence, observed prevalence (also called apparent prevalence), sensitivity, and specificity, respectively (Reiczigel, 2010).

$$prev_{adj} = \frac{prev_{obs} + S_p - 1}{S_e + S_p - 1}$$

All data were statistically analyzed by 'epiR' of statistical programing language R version 3.2 (R development Core Team, Vienna, Austria).

Results and Discussions

Overall, the true seropositivity determined in this study was 89.5%, the highest was found in Phichit followed by Kamphaengphet, Nakhon-Sawan, Uthaithani, Sukhothai and Phitsanulok, respectively (Table 2).

Eight out of 24 serovars were detected including Bratislava, Autumnalis, Djasiman, Javanica, Louisiana, Pyrogenes, Ranarum and Shermani by MAT. Most of the samples (67.2%) were positive to 2 serovars (Fig. 1). For serovars Ranarum and Shermani, the true seropositivity were high in all provinces, especially in Phichit that showed 100% for both serovars. The titration levels of both strains were 1:50 - 1:200. This study

Table 2. True seropositivity and seropositivity in each province from MAT

Provinces	No. of positives/ No. tested	% Seropositivity (95% CI)	% True seropositivity (95% CI)
Kamphaeng Phet	55/58	94.8 (85.6-98.9)	96.4 (87-100)
Nakhon Sawan	46/50	92 (80.8-97.8)	93.4 (82-98.6)
Phichit	50/50	100 (92.9-100)	100 (94.4-100)
Phitsanulok	35/50	70 (55.4-82.1)	70.2 (55.7-81.7)
Sukhothai	41/50	82 (68.6-91.4)	82.9 (69.3-91.6)
Uthai Thani	45/50	90 (78.2-96.7)	91.3 (79.3-97.3)
Total	**272/308**	**88.3 (84.2-91.4)**	**89.5 (85.2-92.9)**

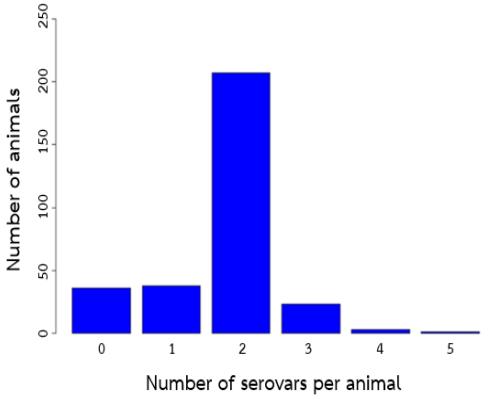

Fig. 1. Number of serovars found in a duck serum samples by MAT method. (n = 308) 0 = No titer against *Leptospira* spp. (11.7%), 1= There are titers against 1 serovar (12.3%), 2= There are titers against 2 serovars (67.2%), 3= There are titers against 3 serovars (7.5%), 4 = There are titers against 4 serovars (1.0%) and 5 = There are titers against 5 serovars (0.3%)

revealed that 88.3% of the ducks included in this study exhibited antibody titer between 1:50 and 1:200 which were lower than those from the study in chickens and ducks fed naturally in Grenada and Trinidad, a Caribbean island with the antibody titer between 1:100-1:400 (Everard et al., 1985). For the latter study, 11% seropositivity against serogroup Shermani and Hebdomadis, and 47% of seropositivity for 2-4 serogroups were also recorded which are consistent with this study that leptospires were found more than 1 serovar per duck.

Serovars Ranarum and Shermani were observed dominantly with seropositivity in the range of 68.1 to 100% and 40.6 to 100%, respectively. Serovar Djasiman was mostly found in Phichit at 23.6%. The seropositivities to serovars Autumnalis, Javanica, Pyrogenes and Louisiana are low (0 - 0.4%) except serovar Pyrogenes in Sukhothai (4.7%) (Table 3). Serovar Ranarum (87%) and Shermani (77%) are the two highest proportions in this study (Fig. 2). Chadsuthi et al. (2017) showed the most predominant serovars for different host species comprising Shermani, followed by Bratislava, Panama, and Sejroe in human; Shermani, Ranarum, and Tarassovi in buffaloes; and Shermani and Ranarum in cattle and pigs. This indicated that serovars Ranarum and Shermani found in free-grazing ducks are similar to those found in livestock and humans.

The samples from healthy flock with highest titer of seropositivity in Phichit province were recollected for *Leptospira* spp. isolation and molecular detection. *Leptospira* spp. could be recultured from blood collected from *L. canicola* inoculated poultries with the highest antibody titer at 1: 2560. In addition, the chickens did not show any symptoms and grow normally until the age of 7 months which consistently with this study (Byrne et al. 1955). In contrast, the study of Gleiser (1955) showed that signs of leptospiremia, e.g. anorexia,

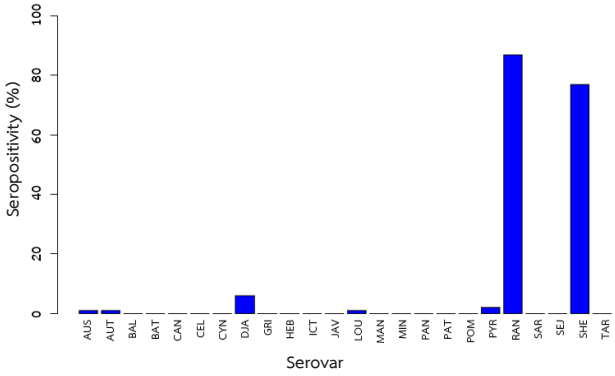

Fig. 2. Proportion of seropositive ducks by MAT serotyping. The proportion is AUS 1%, AUT 1%, DJA 6%, LOU 1% PYR 2% RAN 87% and SHE 77%.

Table 3. Provincial distribution of *Leptospira* serovars

Serovars	% True seropositivity (No. of positives/ No. tested)[a]					
	Kamphaeng Phet	Nakhon Sawan	Phichit	Phitsanulok	Sukhothai	Uthai Thani
AUS					0.4 (2/50)	
AUT			0.0 (1/50)			0.0 (1/50)
DJA			23.7 (13/50)		0.0 (1/50)	2.5 (3/50)
JAV						0.0 (1/50)
LOU			0.4 (2/50)			0.4 (2/50)
PYR	0.0 (1/58)				4.7 (4/50)	0.0 (1/50)
RAN	94.6 (54/58)	91.3 (45/50)	100 (50/50)	68.1 (34/50)	82.9 (41/50)	89.2 (44/50)
SHE	87.3 (50/58)	74.4 (37/50)	100 (50/50)	40.6 (21/50)	72.3 (36/50)	89.2 (44/50)

[a] Value of no. of positives/ no. tested is not related to true seropositivity value.

ruffled feathers and depression, and decrease in hatchability rate were noted in inoculated duck and chicken embryos, respectively. However, no studies in chickens or ducks have been conducted in term of relationship between the level of infective pathogen and shedding of *Leptospira* spp.

In our study, shedding of *Leptospira* spp. in a flock that had the highest seropositivity from MAT was detected by real-time PCR targeting *lipL32*. The real-time PCR positive results from both kidneys (Ct. 39.4) and cloacal swabs (Ct. 36.2-39.8), at 10% and at 47%, respectively, suggested that free-grazing ducks might be a reservoir of Leptospirosis. Detection the genetic materials of pathogenic *Leptospira* spp. using the method of Stoddard (2009) with lower limit of detection (LLOD) of 20 genome equivalents per reaction illustrated 100% sensitivity and specificity. Therefore, in our study, the free-grazing duck might shed at least 20 leptospires. However, the isolation could not be done because of contamination. Moreover, the long transportation and improper preservation might affect the survival of the pathogen (Optimum temperature and pH range are 28-30°C and 7.2-7.6 respectively; Adler, 2014). The experimental research on pathogenesis and transmission of *Leptospira* spp. in free-grazing ducks and possibility of interspecies infection should be conducted for future studies.

Conclusion

This study was a preliminary prevalence survey of leptospirosis in free-grazing ducks in Thailand. The MAT results show overall seropositivity at 88.3% and the two highest prevalent serovars of *Leptospira* spp. were Ranarum 87% and Shermani 77%. In conclusion, free-grazing ducks may be one of the important reservoirs. This information might be useful for livestock officials with regard to control and prevention of animal-to-human transmission.

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