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Prevalence of *Streptococcus suis* in Tonsils of Slaughtered Pigs in Lampang and Phayao Provinces, Thailand, 2009-2010

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Abstract

Streptococcus suis, a swine and human pathogen, may be the cause of high prevalent, sporadic, human cases in northern Thailand. A survey of *S. suis* from the tonsils of slaughtered pigs was conducted in Lampang and Phayao provinces, Thailand, during September 2009-July 2010. Samples of pigs from slaughterhouses of the respective provinces were cultured. *S. suis* was isolated and identified by conventional method and PCR using primers specific to 16S rRNA, and capsular gene types 1(14) and 2(1/2). Additional confirmation was obtained by slide agglutination and capillary tube precipitation with *S. suis* type 1, 2 and 14 antisera. The results of tonsil samples of 236 pigs from Lampang were positive for *S. suis*, *S. suis* type 2, and *S. suis* type 14, at rates of 64.8%, 3.8%, and 2.1%, respectively. Tonsil samples of 559 pigs from Phayao were positive for *S. suis*, *S. suis* type 2, and *S. suis* type 14, at rates of 61.4%, 5.9%, and 1.2%, respectively. There was no significant difference among the prevalence of *S. suis* in pig tonsils and the ambient temperature and humidity ($p > 0.05$). The results of the investigation of virulence-associated genes included suilysin (*sly*), muramidase released protein (*mrp*) and extracellular protein factor (*epf*); of 151 *S. suis* isolates from Phayao, genotypes with *sly*+, *mrp*+ and *epf*+ were detected in 64.9%, 58.9%, and 3.9%, respectively. The high prevalence of *S. suis* carrier in pigs, especially serotypes 2 and 14, and the presence of virulence-associated genes may be risk factors for *S. suis* infection in humans.

Keywords: *Streptococcus suis*, tonsils, pigs, Lampang, Phayao, Thailand

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Introduction

Streptococcus suis is a swine pathogen and has been considered a major problem worldwide in the swine industry. It is also responsible for septicemia and meningitis [1]. The natural habitat of *S. suis* is the upper respiratory tract of

pigs, particularly the tonsils and nasal cavities, as well as the genital and alimentary tracts [2]. The pathogen is a zoonotic agent. Infection in humans was considered to be sporadic among people working with pigs or pork-derived products [3], however the emergence in some countries, such as the outbreak in the People's Republic of China in 2005, where 39 of 215 patients died from *S. suis* infection [4], has changed that perspective. Moreover, it has been described as the most, second-most and third-most common cause of adult meningitis in Vietnam, Thailand, and Hong Kong [5-7], respectively. The clinical signs and symptoms in humans are fever, neck stiffness and impaired consciousness [8]. Some of the most striking effects of *S. suis* meningitis infection are subsequent deafness and/or vestibular dysfunction [8-10]. In Thailand, at least 300 cases of *S. suis* infection have been reported in humans [8,11-16] however, the number of cases seems to have been under-reported. Currently, the number of patients is increasing through awareness and efficient laboratory diagnosis, and most of the cases have accumulated from northern Thailand (information from Bureau of Epidemiology, Department of Disease Control). Since a high prevalence of human cases occurred sporadically in the north, where eating raw pork and pig products is common, a survey of *S. suis* from the tonsils of slaughtered pigs was conducted in Lampang and Phayao provinces.

Materials and methods

Sample collection

During the period September 2009-July 2010, 236 tonsil samples of apparently healthy pigs were collected from slaughterhouses located in 5 districts in Lampang Province, and 559 samples from 9 districts in Phayao Province. Samples from approximately 20 pigs were collected once a week. In instances where a small number of pigs were slaughtered on a particular day, all pigs were sampled. Approximately 180 samples were collected from Phayao Province in three rounds in order to cover all seasons of the year. *S. suis* isolated from one or both tonsils was regarded as positive.

Reference stains

S. suis serotype 1 (NCTC10237) and 2 (NCTC10234) were obtained from National Institute of Animal Health, Japan, *S. suis* serotype 1/2 (2651), and 14 (DAN13730) from Marcelo Gottschalk, Faculté de Médicine Vétérinaire, Université de Montréal, Saint-Hyacinthe, Québec, Canada and *S. suis* serotype 2 strain P1/7 from Anusak Kerdsin, Ministry of Public Health, Nonthaburi, Thailand.

Bacterial isolation and identification

Tonsil samples were cultured onto two media, 5-7% sheep blood agar (oxoid) containing 0.025% oxalinic acid, and Edwards agar (oxoid, UK) containing 5-7% sheep blood, incubated at 37°C under 5% carbon dioxide. Bacterial colonies were examined twice, at 24 and 48 h after cultivation. Typical small transparent alpha- to non-hemolysis colonies were isolated and primary identification was completed using physiological and biochemical tests according to Quinn *et al* [17]. Consequently *S. suis* and capsular genotypes were identified by multiplex PCR using primers specific to 16S rRNA [18], and capsular gene types 1(14) and 2(1/2) [19,20] as described by Worarach *et al* [21]. The primers are shown in Table 1. In summary, DNA templates were prepared from 15-20 colonies of *S. suis* grown on blood agar and washed by suspending in 1 ml of distilled water (DW), then centrifuged at 12,000 rpm for 5 min. The supernatant was discarded and 100 µl of DW was added, then boiled for 10 min and quickly cooled in an ice box. Final centrifuge at 12,000 rpm for 5 min was conducted and the supernatant was then collected. Multiplex PCR per reaction (final volume 25 µl) consisted of 2.5 µl DNA template and 0.2 µM of primer mix (16S rRNA, cps1(14)J and cps2(1/2)J) in multiplex PCR master mix (QIAGEN, USA). Amplification was conducted in a thermal cycler (BIO-RAD, USA). PCR parameters consisted of 35 cycles of 94°C for 45 s, 56°C for 1 min, and 72°C for 1.30 min. PCR amplicons were electrophoresed on 2% agarose gels (CosmoBio Co LTD, Korea) and visualized by UV transillumination after ethidium bromide

Table 1 Primers and expected PCR products.

Primer	Nucleotide (5′-3′)	Expected PCR product (bp)
16S-195(s)	CAG TAT TTA CCG CAT GGT AGA TAT	294[1]
16S-489(as2)	GTA AGA TAC CGT CAA GTG AGA A	
cps1(14)J-(F)	GGC GGT CTA GCA GAT GCT CG	440[2]
cps1(14)J-(R)	GCG AAC TGT TAG CAA TGA C	
cps 2(1/2)J-(F)	CAA ACG CAA GGA ATT ACG GTA TC	675[2]
cps 2(1/2)J-(R)	GAG TAT CTA AAG AAT GCC TAT TG	
epf-F	CGC AGA CAA CGA AAG ATT GA-3′	744[3]
epf-R	AAG AAT GTC TTT GGC GAT GG-3′	
sly-F	GCT TGA CTT ACG AGC CAC AA-3′	248[3]
sly-R	CCG GGC AAT ACT GAT AAG C-3‘	
mrp-F	ATT GCT CCA CAA GAG GAT GG-3‘	188[3]
mrp-R	TGA GCT TTA CCT GAA GCG GT-3′	

[1] Marois *et al* [18]; [2] Smith *et al* [20]; [3] Smith *et al* [19]

staining. Finally, the serotype was confirmed by precipitation in capillary tubes and slide agglutination, as described by Pathanasophon *et al* [22].

Detection of virulence-associated genes

Amplification of virulence-associated genes, suilysin (*sly*), muramidase released protein (*mrp*) and extracellular protein factor (*epf*), was achieved by using multiplex PCR according to Silva *et al* [23] with minor modification as described by Pathanasophon *et al* [24]. Primers are presented in Table 1. In summary, DNA templates were prepared as described previously, and multiplex PCR per reaction (final volume 25 µl) consisted of 2.5 µl DNA template and 0.2 µM of primer mix in multiplex PCR master mix (QIAGEN, USA). Amplification was conducted in a thermal cycler (BIO-RAD, USA). PCR parameters consisted of 35 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1.30 min. PCR amplicons were electrophoresed and visualized as described above. *S. suis* serotype 2 strain P1/7 and distilled water served as positive and negative controls, respectively, and compared with 100 bp DNA Ladder (Fermentas, Germany).

Statistical analysis

Two-tailed Pearson's Correlation and linear regression analysis were used to investigate the correlation among the prevalence of *S. suis* in pig tonsils and the ambient temperature and humidity data from Meteorology Department of Thailand.

Results

The results for the *S. suis* isolated from the tonsils of pigs slaughtered in 5 districts of Lampang and 9 districts of Phayao provinces are shown in Table 2. The prevalence of *S. suis*, *S. suis* Type 2, and *S. suis* Type 14, from Lampang Province was 64.8%, 3.8%, and 2.1%, respectively. The highest prevalence of *S. suis* and *S. suis* Type 2 were found in Hang Chat District (75.6% and 9.8%, respectively), while *S. suis* Type 14 was highly prevalent in Muang District (3.3%). The prevalence of *S. suis*, *S. suis* Type 2, and *S. suis* Type 14, in Phayao Province was 61.4%, 5.9%, and 1.2%, respectively. The highest prevalence of *S. suis* was found in Phu Kamyao (76.7%), *S. suis* Type 2 in Dok Khamtai (11.7%), and *S. suis* Type 14 in Mae Chai and Dok Khamtai districts (5.0%). Table 3 shows the isolation frequency of *S. suis*, *S. suis* Type 2, and *S. suis* Type 14, in

Table 2 Results of *S. suis*, *S suis* Type 2, and *S suis* Type 14, isolation from the tonsils of pigs slaughtered in Lampang and Phayao provinces.

Province/district	No. of pigs	No. of positive (%)		
		S. suis	*S. suis* Type 2	*S suis* Type 14
Lampang				
Mueang	60	39 (65.0)	2 (3.3)	2 (3.3)
Chae Hom	42	27 (64.3)	3 (7.1)	1 (2.4)
Ngao	39	22 (56.4)	0 (0.0)	0 (0.0)
Hang Chat	41	31 (75.6)	4 (9.8)	1 (2.4)
Thoen	54	34 (62.9)	0 (0.0)	1 (1.9)
Total	236	153 (64.8)	9 (3.8)	5 (2.1)
Phayao				
Mueang	140	75 (53.6)	11 (7.9)	1 (0.7)
Mae Chai	60	41 (68.3)	2 (3.3)	3 (5.0)
Phu Kamyao	30	23 (76.7)	2 (6.7)	0 (0.0)
Dok Khamtai	60	37 (61.7)	7 (11.7)	3 (5.0)
Chiang Kham	60	35 (58.3)	3 (5.0)	0 (0.0)
Phu Sang	60	38 (63.3)	3 (5.0)	0 (0.0)
Pong	60	43 (71.7)	2 (3.3)	0 (0.0)
Chun	49	29 (59.2)	1 (2.0)	0 (0.0)
Chiang Muan	40	22 (55.0)	2 (5.0)	0 (0.0)
Total	559	343 (61.4)	33 (5.9)	7 (1.2)

the tonsils of slaughtered pigs in Lampang and Phayao provinces, elucidated by month, along with data of ambient temperature and humidity from the Meteorological Department of Thailand. The highest prevalence of *S. suis*, *S. suis* Type 2, and *S. suis* Type 14, in the tonsils of slaughtered pigs were found in January, May, and July, at 76.7%, 18.8%, and 5.3%, respectively, while the lowest temperature (15 °C) and humidity (58%) were reported in February and March. There was no correlation among the prevalence of *S. suis* in pig tonsils and the ambient temperature and humidity data, by two-tailed Pearson's Correlation and linear regression analysis ($p > 0.05$). Table 4 shows the results of virulence-associated-gene investigation, including *sly*, *mrp* and *epf* from 151 *S. suis* cases isolated from 180 pigs from 9 districts of Phayao. The results revealed that most strains were positive for *sly* (64.9%), followed by *mrp* (58.8%), and *epf* (3.9%). Strains with genotype *mrp+sly+* showed a high prevalence, at 43.7%.

Discussion

We have observed high carrier rates of *S. suis* in pigs brought to slaughterhouses in Lampang and Phayao provinces (64.8% and 61.4%, respectively). These percentages are higher than those found in our previous survey in eastern and western Thailand (14.3%), and southern Vietnam (41%) during 2006-2007. In addition, *S. suis* type 2 was found to be at higher prevalence (3.8% and 5.8% *vs* 0%), while 8% was reported in southern Vietnam [22, 25]. Interestingly, serotype 14 was also detected in this study, but not found in eastern or western Thailand [22]. The relatively lower prevalence of *S. suis* type 14 was coincidental with human infections. According to Kerdsin *et al* [26], of 177 human *S. suis* isolates in Thailand,

Table 3 Isolation frequency of *S. suis*, *S. suis* Type 2, and *S. suis* Type 14, in the tonsils of slaughtered pigs in Lampang and Phayao provinces, elucidated by month, and data for ambient temperature and humidity from the Meteorological Department of Thailand.

Month/Year	No. of pigs	% of Positive			Temperature			Average humidity
		S. suis	type 2	type 14	Max	Min	Mean	
6/2009 (L)	63	52.4	6.4	0.0	32	24	27	79
7/2009 (L)	95	73.7	3.2	5.3	32	24	27	80
8/2009 (L)	78	66.7	2.6	0.0	32	24	27	82
9/2009 (P)	80	53.8	3.8	5.0	32	24	27	83
10/2009 (P)	80	47.5	2.5	0.0	32	23	26	84
11/2009 (P)	20	35.0	0.0	0.0	31	18	23	77
1/2010 (P)	60	76.7	6.7	0.0	31	17	22	75
2/2010 (P)	79	73.4	0.0	0.0	34	15	23	61
3/2010 (P)	40	52.3	0.0	0.0	35	19	26	58
5/2010 (P)	80	60.0	18.8	0.0	36	25	30	66
6/2010 (P)	80	65.0	5.0	0.0	33	25	29	70
7/2010 (P)	40	55.0	2.5	0.0	33	25	28	71

L: Lampang Province, P: Phayao Province

Table 4 Virulence-associated phenotypes of *S. suis* isolated from tonsils of 180 pigs slaughtered in 9 districts of Phayao Province, January-March 2010.

No. of *S. suis* strains	No. of strain(s) with virulent-associated gene(s) positive (%)						
	sly+	*mrp+*	*mrp+sly+*	*mrp+sly+epf+*	*A.sly+*	*A.mrp+*	*A.epf+*
151	26 (17.2)	17 (11.2)	66 (43.7)	6 (3.9)	98 (64.9)	89 (58.9)	6 (3.9)

6.8% belonged to serotype 14, and 11 of 12 isolates were isolated from humans in northern Thailand. The clinical features in patients infected with *S. suis* type 2 and type 14 were similar, but there were no fatal cases from *S. suis* type 14. Infection of *S. suis* in pigs can occur at an early stage of life through vertical transmission from carrying sows [27,28] but it is usually transmitted nasally or orally and then colonizes the palatine tonsils of both clinically ill and apparently healthy pigs [29,30]. The prevalence of *S. suis* and *S. suis* type 2 seems to increase in January (76.7% and 6.7%, respectively) when mean ambient temperature decreases (22 °C). We therefore collected samples for two more rounds in order to cover all seasons of the year; there was, however, no statistical relation ($p > 0.05$). Human infection cases peaked during the rainy season of 2006-2008 but transmission was associated with only adult behavior, as there were no cases of infection in children [31]. Interestingly, most *S. suis* harbored in the tonsils of slaughtered pigs in Phayao Province possessed virulence-associated genes, *sly+* (64.9%), *mrp+* (58.8%) and *epf+* (3.9%) and *mrp+sly+* (43.7%). The figures are similar to those found in diseased pigs in our previous study (70.2%, 51.1%, 6.4%, and 38.3%, respectively). While healthy slaughtered pigs had much lower figures of *sly+* (29.4%), *mrp+*

(26.4%) and *epf*+ (0%) [24], the pigs that harbored *S. suis* possessed virulence-associated genes in their tonsils without showing signs of being ill. This would indicate that these animals were resistant to invasive disease, possibly through an immune mechanism. The *S. suis* with virulence-associated genotypes were presumed to be correlated with invasion. The experiment by Tan *et al* [32] reported that after 48 h post-infection in swine with *S. suis* type 2, *epf* and *sly* were highly expressed in bacteria isolated from the lungs and brain, and were differentially expressed in a time-specific or an organ-specific manner *in vivo*. In humans, Kerdsin *et al* [31] reported *S. suis type 2* with virulent-associated phenotypes in 165 cases, 88% (36/42) of the isolates from CSF and 53% (66/124) from blood had the *mrp+epf+sly+* genotype. The other profiles *mrp-epf-sly+* or *mrp+ epf-sly-* or *mrp*+ epf- sly-* were isolated from blood. It is claimed that *S. suis* type 2 carriers in pigs are capable of causing human infection [3, 25], and that consumption of raw pork or pig blood in addition with underlying diseases, such as alcohol abuse, diabetes, valvular heart disease, *etc*, are risk factors of *S. suis* infection in humans.

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