SURVEY OF LEPTOSPIROSIS OF SMALL MAMMALS IN THAILAND

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Abstract. During 1999-2000, kidney tissues of approximately 15% of 1,310 rodents trapped from northeastern provinces of Thailand were tested for the presence of leptospires. Our direct immunofluorescent assay (DFA) for detection of leptospires showed 100% sensitivity and 94% specificity with the culture data. Both methods identified *R.norvegicus* as the highest source of infection. Among isolated *Leptospira*, 137 were serotyped by cross agglutinin absorption and/or a microscopic agglutination, and gave some variations and similarities at the serovar level to the DFA results. DFA data demonstrated over half of the positive animals were infected with several serovars of *Leptospira interrogans*. A subsequent DFA study in Bangkok in 2002 revealed leptospiral infection in 33% of 42 rats and shrews. The most common infecting serovars were Autumnalis and Canicola identified in rural and urban animals, respectively. This finding suggests that wild small mammals may act as important sources of pathogenic leptospires and warrant active surveillance to understand the epidemiology of transmission and control of carrier animals.

INTRODUCTION

Leptospirosis is a significant infection in domestic and wild animals (Farr, 1995; Faine *et al*, 1999; Ko *et al*, 1999). Pathogenic *Leptospira* produce a wide spectrum of clinical and subclinical manifestations in humans, and infection occurs after contact with the urine of carrier animals or a contaminated environment. Between 1995 and 1999, an increase in the incidence of fatal human leptospirosis cases was reported from 143 to 6,080 cases in our country (Hinjoy, 2000). The majority of the affected cases were people or farmers in rural Thailand (Montienarsana *et al*, 1997; Tangkanakul and Kingnate, 1998) but the precise source of infection was underinvestigated. Various species of wild and domestic mammals were reported as maintenance hosts, carrying diverse types of pathogenic leptospira (Everard *et al*, 1995; Vinetz *et al*, 1996; Heisey *et al*, 1998; Bunnell *et al*, 2000). In central Thailand, Bataviae and Javanica were the most common serotypes found in rats (Boonpucknavig *et al* 1965; Sundharagiat *et al*, 1965). Bataviae, Javanica, Canicola and Bangkok were more common in dogs, while Pomona was common in swine (Sundharagiat *et al*, 1965). Over 30 years later, Bataviae and Javanica still exists in wild rodents and domestic animals, including dogs, cats, pigs, and cows, in urban and provincial Thailand (Heisey *et al*, 1998). While others reported a diversity of leptospires in mammals, such as Bratislava and Grippotyphosa in canine (Scanziani *et al*, 2002), and Sejroe, Icterohaemorrhagiae and Brasiliensis in the Indian mongoose (Tomich, 1979; Everard *et al*, 1980). For a better understanding of the true source of leptospires of human relevance, kidney tissue of rodents eliminated during out-

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breaks in rural provinces in 1999-2000 and in Bangkok by 2002 were studied by culture isolation and direct immunofluorescent assay (DFA).

MATERIALS AND METHODS

Rodents and culture isolation

Animal experiments were approved by the animal research committee of the National Laboratory Animal Center.

Two groups of small mammals were live captured and the species were identified by zoologists of the Thai Agricultural Zoology Research group. The first group consisted of 976 *Rattus* spp, 316 *Bandicota* spp and 18 mice (*Mus* spp) randomly trapped in five provinces of northeast Thailand between June 1999 and September 2000. The second group of 35 *Rattus* spp and 7 shrews (*Suncus* spp) were obtained from eight different urban districts of Bangkok from August to September 2002. All the animals were euthanasized using carbon dioxide gas with immediate removal of the internal organs, including the kidney. A half the kidney specimen was minced using a tissue grinder and cultured in semisolid Ellinghousen-McCullough-Johnson-Harris (EMJH) medium and incubated at 28-30ºC in the dark (Johnson and Harris, 1967). Cultures were examined weekly for 10 weeks using a dark field microscope. Samples with organisms were considered positive. The obtainable isolates were then serotyped by a cross agglutinin absorption test (CAAT) and/or a microscopic agglutination test (MAT) as previously described (Dikken and Kmety, 1978; Sulzer and Jones, 1978). The remaining portion of the kidney was snap frozen and kept at -70ºC until tested by direct immunofluorescent assay (DFA).

Bacteria

Twenty-three reference strains of leptospires obtained from the National Leptospirosis Reference Center, National Institute of Health, Thailand were used as reference antigens for MAT. The leptospira were periodically checked using specific reference antisera obtained from the Centers for Disease Control and Prevention (CDC), Georgia, United States of America and the WHO/FAO/OIE Collaborating Center for Reference and Research on Leptospirosis, Australia. The eleven most common pathogenic serovars for Thailand and one non-pathogenic serovar *L. biflexa*, were used as immunizing antigens in rabbits as whole live leptospires after 5-7 days growth in liquid neopeptone medium (Table 1).

Specific antisera and fluorescent conjugates

Rabbit hyperimmune sera were raised individually against the following 12 serovars: Australis, Autumnalis, Bangkok, Bataviae, Canicola, Grippotyphosa, Hebdomadis, Icterohemorrhagiae, Javanica, Pomona, Pyrogenes and Patoc by weekly intravenous injection for 4-6 weeks (Sitprija *et al*, 1980). The MAT was used to detect antibody titers to live leptospiral cultures on microtiter plates (Cole *et al*, 1973). Antisera with high agglutinating titers (of >12,800) were collected and used in conjugation. Briefly, serum globulins were fractionated by ammonium sulphate precipitation and labelled with fluorescein isothiocyanate (FITC) dye (Nairn, 1976). Unbound proteins and excess free dye were removed by Sephadex gel filtration and tissue absorption. The resulting fluorescein-labelled antibody conjugates were predetermined and optimized with smears of reference cultures before use. The reactivity and specificity of the test was determined at a final dilution of conjugate that gave a strong fluorescence with target antigens of the homologous leptospires and no staining with the heterologous or unrelated strains particular to the different serogroups.

Identification of leptospires

To identify leptospire infection, kidney tissues were cyosectioned at 4-5 µ thick and fixed in cold acetone for 5 minutes before drying at room temperature. Sections were stained with appropriate dilutions of individual fluorescent conjugates for 30 minutes. After 15 minutes of washing off the excess conjugate, the sections were mounted and examined under a fluorescent microscope (Fluophot, Japan) equipped with a filter set for FITC. A positive finding on DFA of kidney revealed a yellowish-green fluorescence to the spiral leptospirae which was distinquishable from the dark background of the surrounding tissues.

The isolates recovered were serotyped by MAT at the local laboratory and compared with

the results of the cross agglutinin absorption test (CAAT) of the corresponding isolates carried out at the WHO/FAO/OIE Collaborating Center for Reference and Research on Leptospirosis, Australia.

RESULTS

From the initial study of rural rodents, leptospires were isolated in 190 of 1,310 (15%) trapped animals. Of the 9 different rodent species obtained, the infection rates varied from a high of 41% for the 307 *R. norvegicus* to none in Mus spp (Table 2).

To determine the detection limit of the DFA using fluorescent conjugates of local preparation, the positivity of the test was considered by specific reactivity to the aggregates or scattered leptospiral bacteria at the luminal surface of the proximal renal tubules, demonstrated as yellowish-green fluorescence (Figs 1A-1B). The sensitivity, specificity, positive (PPV) and negative predictive values (NPV) of the DFA test were 100, 94, 98 and 100%, respectively (Table 3A). The DFA identification of leptospiral infection was evident in 88 out of the 119 kidney samples of randomly selected negative and positive rodents.

Table 1
Serogroups and serovars of *Leptospira interrogans* and non pathogenic *Leptospira biflexa* used in this study.

Serogroups	Serovars	Reference strains
L. interrogans		
1. Australis	Australis[a]	Ballico
	Bangkok[a]	Bangkok D92
	Bratislava	Jez Bratislava
2. Autumnalis	Autumnalis[a]	Akiyami A
	Rachmati	Rachmat
3. Ballum	Ballum	Mus 127
4. Bataviae	Bataviae[a]	Swart
5. Canicola	Canicola[a]	Hond Utrecht IV
6. Celledoni	Celledoni	Celledoni
7. Cynopteri	Cynopteri	3522 C
8. Djasiman	Djasiman	Djasiman
9. Grippotyphosa	Grippotyphosa[a]	Moskva V
10. Hebdomadis	Hebdomadis[a]	Hebdomadis
11. Icterohemorrhagiae	Icterohemorrhagiae[a]	RGA
	Copenhageni	M 20
12. Javanica	Javanica[a]	Veldratbatavia 46
13. Manhao	Manhao	Ll 130
14. Pomona	Pomona[a]	Pomona
15. Pyrogenes	Pyrogenes[a]	Salinem
	Zanoni	Zanoni
16. Sarmin	Sarmin	Sarmin
17. Sejroe	Sejroe	M 84
	Hardjo	Hardjoprajitno
18. Shermani	Shermani	LT 821
19. Tarassovi	Tarassovi	Perepelicin
L. biflexa		
20. Semaranga	Patoc[a]	Patoc 1

[a] Leptospires used for rabbit immunization

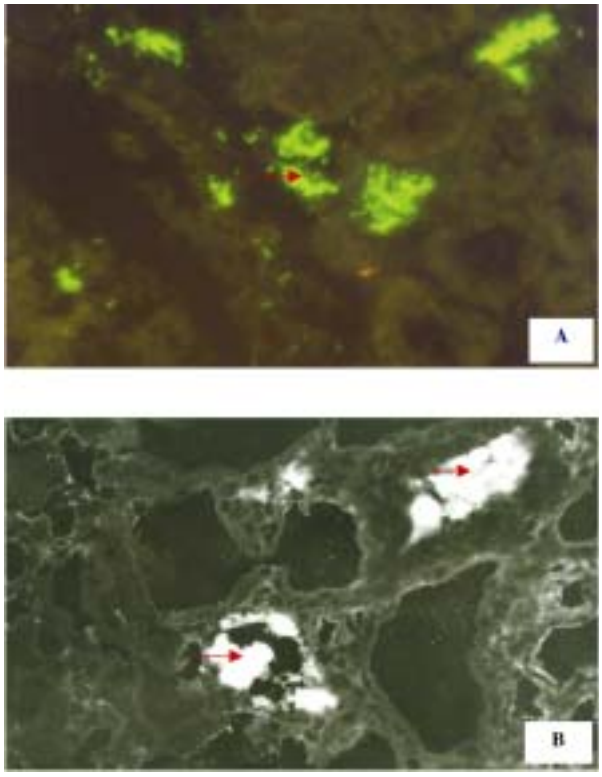

Fig 1–Proximal and distal lumens stained by fluorescein labelled anti-leptospiral antibodies against serovar bataviae (A) or autumnalis (B). Positive reactivity (arrows) is bright yellowish -green fluorescence or whitish appearance on black and white film (below). Magnification x 200.

Only two had false-positive results. All the DFA positive cases were found reactive for single, double, triple or more different serovars in the kidneys with frequencies of 24, 19 and 57%, respectively (Table 3B).

The isolates were typed to the serovar level by conventional CAAT and/or MAT, which demonstrated some similarities and differences to the results of DFA (Table 4). Of 190 isolates, 137 were determined to be Pyrogenes (49%), Bataviae (31%), Autumnalis (13%), Australis (4%) or Javanica (3%). Both the DFA and serotying methods had the same findings for Autumnalis, Australis and Bataviae, while the other results did not coincide.

An additional group of small mammals sur-

Table 2
Species and number of wild animals positive for *Leptospira interrogans* (Results in parentheses as No. positive / animals studied).

Rodent	% positive isolates rural source	% positive DFA urban source
1. *R.norvegicus*	41 (127 /307)	63 (12/19)
2. *B.indica*	14 (36 /265)	-
3. *R.rattus*	5 (10 /184)	6 (1/16)
4. *R.losea*	9 (6 /71)	-
5. *R.argentiventer*	6 (6 /98)	-
6. *B.savilei*	6 (3 /51)	-
7. *R.exulans*	1 (2 /316)	-
8. *Mus cervicolor*	0 (0/12)	-
9. *Mus calori*	0 (0/6)	-
10. *Suncus murinus*	-	14 (1/7)
Total	15 (190 /1,310)	33 (14/42)

Table 3A
Sensitivity and specificity of DFA compared to kidney isolation of leptospires in 119 specimens tested.

	Isolation method		
	Positive	Negative	Total
DFA method			
Positive	86	2	88
Negative	0	31	31
Total	86	33	119
% Sensitivity	100		
% Specificity	94		
% PPV	98		
% NPV	100		

Table 3B
Results of 88 rodents positive leptospires with variable serovars involved.

Serovars encountered	% (No.) positive DFA
Single	24 (21)
Double	19 (17)
Multiple (triple or more)	57 (50)

Table 4
Relative % frequencies (No. positive) of outcome serovars detectable in kidneys of urban and rural animals.

Serovar identification	[a]Urban (n =14)	[a]Rural (n = 88)	[b]Rural (n =137)
Autumnalis	43 (6)	77 (68)	13 (18)
Australis	-	15 (13)	4 (6)
Bangkok	64 (9)	61 (54)	0
Bataviae	86 (12)	43 (38)	31 (42)
Canicola	93 (13)	41 (36)	0
Grippotyphosa	-	13 (11)	0
Hebdomadis	43 (6)	49 (43)	0
Icterohemorrhagiae	-	19 (17)	0
Javanica	43 (6)	-	3 (4)
Pyrogenes	43 (6)	-	49 (67)
Pomona	-	52 (46)	0
Patoc	-	0	0

[a]Serovars identified by DFA of the kidneys
[b]Serovars identified by CAAT and /or MAT of the isolates
-Not done

veyed from various non-endemic areas of Bangkok was studied. DFA was the only method used to determine the presence of leptospires in the kidney tissues. Of 42 rats and shrews, only 14 (33%) were positive by DFA. Table 4 shows the data for the DFA method, Canicola (93%) and Autumnalis (77%) were the predominant serovars encountered in urban and rural animals, respectively.

DISCUSSION

The epidemiology and true incidence in hosts of leptospirosis are likely underinvestigated and difficult to assess. This is because of the unavailability of an appropriate test, given the time-consuming cultivation method and a lack of clinical index of suspicion. Small mammals were found to be carrying pathogenic leptospires related to the current outbreak in Thailand. Wild animals in these areas were investigated by culture isolation and locally developed DFA. Infecting leptospires in the kidneys were sufficiently detected with specific fluorescein-antibody conjugates against the serovars of local interest. Intense fluorescence was observed at the luminal surface of proximal tubules where aggregated leptospiral organisms localized. Both DFA and isolation methods showed that *R. norvegicus* was a predominant host for pathogenic *Leptospira* spp. The obtainable isolates of rural rodents were typed to the serovar level by CAAT and/or MAT. No previous studies have reported comparing infecting leptospires in host tissues with the serological results of the corresponding isolates. Our findings show some similarities and differences regarding the specific serovars depending on the method used. These observations may result from the technical differences as a competition effect with the cultivation of a mixed population of leptospiral isolates. Microorganisms may fail to grow due to difficulty in the propagation of some leptospira with different nutritional needs and specific conditions needed (Faine, 1998). A mixed population of leptospires was detected in over a half of the DFA positive animals studied. Our DFA test system did not show cross-reaction with any leptospires of other serogroups, resulting in approximately 24% of the positive animals reactive with a single serovar. DFA should be a valuable method for the detection of infection in comparison to the laborious method of the culture isolation of animal leptospirosis.

Serotypes in the current study were somewhat different from previous reports. A shift in the serovars was found from Bataviae and Javanica to Canicola and Bataviae from rats in urban Bangkok and to Autumnalis from rats in rural Thailand. Overall, the current estimate of leptospirosis infection in wild rodents was different from the seroprevalence in other reports (Heisey *et al*, 1998; Kollars *et al*, 2002; Wangroongsarb *et al*, 2002; Kositanont *et al*, 2003). The reason for this discrepancy is not clear. One factor is that the serology to diagnose leptospirosis is complicated (Theirmann and Garrett, 1983; Faine *et al*, 1999). Another factor could be the underestimation of the incidence (Sasaki *et al*, 1993; Kollars *et al*, 2002).A DFA study done in 2002 showed a two times higher rate (33%) of infection in wild animals in urban than rural groups (15%). People seropositive to leptospires after environmental exposure

often had asymptomatic infection (Phraisuwan *et al*, 2002). Patients living in Bangkok having no history or a low index of suspicions for exposure have been found in sporadic leptospirosis cases (Ariyapruchya *et al*, 2003). Both these factors suggest the importance of animal carriers, especially *R. norvegicus*, in the transmission of *Leptospira* pathogenic to humans (Sundharagiat *et al*, 1965; Everard *et al*, 1995; Vinetz *et al*, 1996). The DFA data suggest small mammals are a source of multiple leptospiral serovars. This had become a major concern regarding the transmission and need for a greater awareness of environmental contamination of the region.

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