

## Infection rate of *Leptospira interrogans* in the field rodent, *Apodemus agrarius*, in Korea

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### SUMMARY

Leptospirosis has significantly decreased in Korea since 1988, following the leptospiral vaccination programme initiated in 1988. Whether this wholly explains the decreased incidence is uncertain. As an initial step to answer this question, infection rates of *Leptospira interrogans* in field rodents, *Apodemis agrarius*, were examined and compared with previous data.

Two hundred and twenty-two *A. agrarius* were captured during October–December 1996. Spirochaetes were isolated from 22 (9.9%) and leptospiral DNA was detected in an additional 6 rodents (12.6%). Subsequent microscopic agglutination tests (MAT) classified all these isolates as *L. interrogans* serogroup Icterohaemorrhagiae serovar *lai*. The above data did not significantly differ from previous surveys in 1984–7. There was no significant change of *L. interrogans* infection in field rodents following the introduction of the vaccination programme in Korea. Further studies are needed to determine the role of human vaccination in reducing incidence.

### INTRODUCTION

Leptospirosis has emerged as one of the major public health problems in Korea since 1984 when the first case was reported [1]. Serological evidence of the widespread occurrence of leptospirosis has been reported [2, 3]. Since 1989, the number of cases diagnosed serologically and seropositive rates among patients with acute febrile episodes has decreased significantly [2]. The reason for the decrease has not been identified, but the launch of the nationwide vaccination programme against leptospirosis may be a possible explanation. Vaccine production with a local strain had been licensed. Immunization of the high risk groups in rural areas began in 1988.

Since 1984, the aetiologic agent of leptospirosis,

*Leptospira interrogans*, has been isolated from various sources such as patients and wild rodents [4]. During 1984–95, a total of 80 cultures of *L. interrogans* were isolated and identified in Korea. From field rodents, especially *Apodemus agrarius*, 68 cultures were isolated during 1984–8 [4]. All isolates from field rodents were identified as serogroup Icterohaemorrhagiae. Among them, 46 isolates were subjected to serovar identification and 40 isolates were classified as serovar *lai* and one as serovar *hongchon* [4].

As an initial step to explain the decreased incidence of leptospirosis in Korea, the infection rate of *L. interrogans* in *A. agrarius*, the most important reservoir in Korea, was investigated by culture and PCR detection of leptospire. Furthermore, the serogroup and serovar were investigated and compared to previous reports.

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## MATERIALS AND METHODS

### Bacterial strains and antibodies

Fifteen serogroup reference strains of *L. interrogans*, 15 serovar reference strains in serogroup Icterohaemorrhagiae and 22 newly isolated strains in this study were propagated in Ellinghausen McCullough Johnson Harris (EMJH) medium at 30 °C [5]. Antisera were prepared by inoculating 1, 2, 4, 5, 5 ml of leptospiral culture ( $2 \times 10^8$  ml<sup>-1</sup>) respectively at 1-week intervals into rabbits [6]. Ten days after the fifth immunization, rabbits were bled and antisera were obtained. The serovar typing monoclonal antibody panel was obtained from WHO/FAO Collaborating Center for Reference and Research on Leptospirosis, Amsterdam, the Netherlands.

### Collection of *A. agrarius* and harvesting of the organs

The field rodents were collected by the modified Sherman live traps [7] at 16 different areas in 5 provinces of Korea in October–December 1996 (Fig. 1, Table 1). The live rodents were identified on the basis of their morphology, anaesthetized by chloroform, dissected and then their kidneys were harvested. The harvested organs were stored in liquid nitrogen until analysis.

### Culture

Isolation of *L. interrogans* was performed as described previously [8]. Briefly, ground tissues were suspended with EMJH media (5%, v/v), inoculated into EMJH media and cultured for 8 weeks with dark field microscopic monitoring for the leptospiral growth every 5 days.

### Polymerase chain reaction (PCR)

The frozen tissues were thawed and sectioned into the appropriate size (50 mg). Sectioned tissues were soaked in 80 µl of PBS in eppendorf tubes and ground by using a compatible pestle. DNAs were extracted from ground tissues (25 mg) by the spin column method (QIAamp Tissue Kit, Qiagen Inc. Germany) as described in manufacturer's instructions. The PCR was performed as described by Gravekemp and colleagues [9] with some modification. The nucleotide sequences of forward primer, G1, and reverse primer, G2, were 5'-CTGAA TCGCT GTATA AAAGT-3'

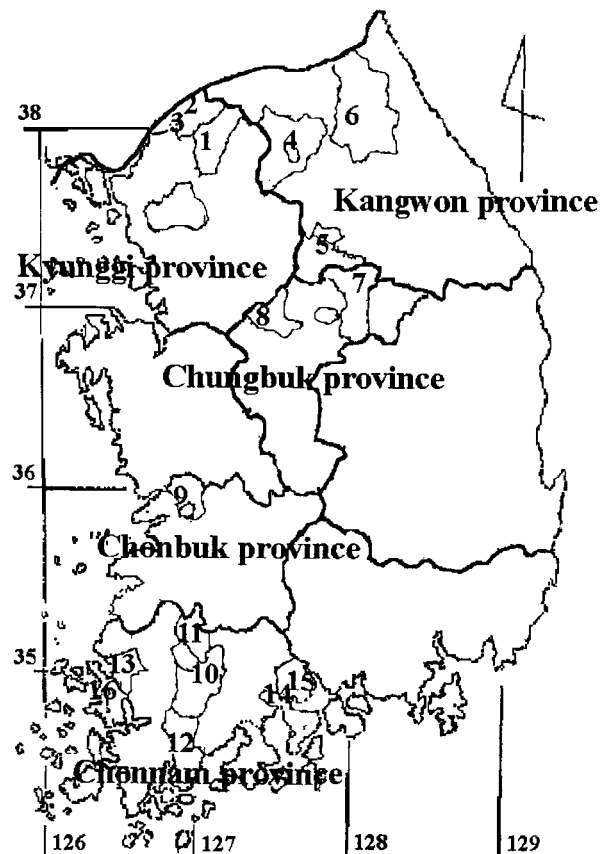


Fig. 1. Field rodent collection sites indicated by numbers. The number of collected rodents and area name were described in Table 1.

and 5'-GGAAA ACAA TGGTC GGAAG-3' respectively. The PCR using these G1 and G2 primers could detect most of pathogenic leptospires [11]. The PCR amplification mixture (total volume, 100 µl) containing 10 mM Tris-HCl (pH 7.4), 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.1% Triton X-100, 200 µM each dNTPs, 5 pmol primers G1 and G2, 2.5 Unit of Taq polymerase (AmpliTaq, Perkin-Elmer-Cetus, USA), and 10 µl of template DNA was denatured at 95 °C for 30 s, annealed at 50 °C for 30 s, and then the chain was extended at 72 °C for 1 min in a thermal cycler (Thermocycler 9600, Perkin-Elmer Cetus, USA). This cycle was repeated 30 times. The presence and size of the amplified products, about 285 bp, was determined by 2% agarose gel electrophoresis containing ethidium bromide.

### Identification of serogroup and serovar by microscopic agglutination test (MAT)

Serogroup and serovar of isolates were identified by reactive patterns of these isolates with antisera to

Table 1. Isolation and PCR confirmation of *L. interrogans* from *A. agrarius*

Collection sites	No. of rodents examined	No. of isolates positive (%)	PCR detection positive (%)
Central Korea	76	11 (14.5)	12 (15.8)
Kyunggi province	33	6 (18.2)	7 (21.2)
1. Pochon	26		
2. Yonchon	3		
3. Jongok	4		
Kangwon province	34	4 (11.7)	4 (11.7)
4. Chuncheon	28		
5. Wonju	2		
6. Inje	4		
Chungbuk province	9	1 (11.1)	1 (11.1)
7. Jechon	2		
8. Umsong	7		
Southern Korea	146	11 (7.5)	16 (10.9)
Chonbuk province	31	1 (3.2)	2 (6.5)
9. Iksan	31		
Chonnam province	115	10 (8.7)	14 (12.2)
10. Whasun	30		
11. Damyang	42		
12. Janghung	5		
13. Hampyong	6		
14. Suncheon	13		
15. Kwangyang	5		
16. Muan	14		
Total	222	22 (9.9)	28 (12.6)

reference strains and typing monoclonal antibodies using MAT as described by Cole and colleagues [10].

## RESULTS

### Isolation and PCR detection of *L. interrogans*

A total of 222 field rodents were captured in October–December 1996. Among them, 76 rodents were captured at 8 areas in middle part (Kyunggi, Kangwon and Chungbuk provinces) and 146 rodents were at 8 areas in southern part (Chonnam and Chonbuk provinces) of Korea (Fig. 1, Table 1). All the rodents captured was identified as *Apodemus agrarius*.

Spirochetes were isolated from 22 rodents using the conventional *L. interrogans* isolation method (9.9% of isolation rate). Geographically, 11 isolates were from the rodents in central Korea and another 11 from southern part of Korean peninsula. The isolation rates were 14.5% (11/76) and 7.5% (11/146) respectively (Table 1). The infection rate of *L. interrogans* in *A. agrarius* seemed to be somewhat higher in

central than in southern Korea although the difference was not statistically significant.

Leptospiral DNA was detected in the extracted DNAs from 28 rodents with the leptospira specific PCR detection method using G1, G2 primers [9]. PCR positive rodents included all rodents from which spirochaetes were isolated. The PCR detection rate was 15.8% (12/76) in central and 10.9% (16/146) in southern Korea (Table 1).

### Identification of serogroup and serovar

Serogroup of 22 isolates were identified by reactive patterns to hyperimmunesera to reference serogroup strains in *L. interrogans* using MAT (Table 2). All isolates showed 100% and 25% cross reactivity to two serovars, *lai* and *copenhageni* respectively, in serogroup Icterohaemorrhagiae. However, cross reactivities to strains in other 15 serogroup in *L. interrogans* tested were less than 3% except for serogroup Canicola (6.25%).

Serovar identification was done by MAT using seven monoclonal antibodies. Immunizations with *L.*

Table 2. Serogroup and serovar identification of isolates

Antisera		Cross reactivity with isolates (%)						
Serogroup	Serovar (strain)	All isolates						
Icterohaemorrhagiae	<i>lai</i> (017)	100						
Icterohaemorrhagiae	<i>copenhageni</i> (M20)	25						
Canicola	<i>canicola</i> (Hond Utrecht IV)	6.25						
Other serogroup		< 3						
Serovar (Strain)	MAb...	F20-C4 -3	F52C2 -2	F70C7 -3	F70C20 -2	F82C1 -3	F82C2 -2	F89C3 -3
All 22 isolates in this study		-	-	++++	-	++	++++	-
<i>lai</i> (HY10*)		-	-	+++	-	++	++++	-
<i>lai</i> (017†)		-	-	++++	-	++	++++	-
<i>yeonchon</i> (HM3*)		++	++	+++	++	++	-	+++
<i>hongchon</i> (18R*)		++++	-	+++	-	-	++++	-

Serogroup of isolates was identified by cross-reactivity of isolates with antisera to serogroup reference strains. Cross reactivity (%) = (reciprocal titre with isolates/reciprocal titre with homologous strain) × 100.

Serovar was identified by reactive patterns to monoclonal antibodies. All reactivities were measured by microscopic agglutination test (MAT).

\* Reference strain isolated in Korea [11–13].

† Reference strain obtained from NIH Japan [14].

Table 3. *L. interrogans* isolated from field rodents in Korea

Isolation*					
Year	No. of rodents examined	No. of isolation	Isolation rate	Identification† serogroup/serovar (no. of identified strains)	Reference
1984	45	7	15.5	Icterohaemorrhagiae/ <i>lai</i> (4)	Isolation rate [3] Identification [15–17]
1985	295	44	14.9	Icterohaemorrhagiae/ <i>lai</i> (24) Icterohaemorrhagiae/ <i>hongchon</i> (1) Icterohaemorrhagiae/unidentified (1)	Isolation rate [3] Identification [11, 15–17]
1986	247	4	1.6	Icterohaemorrhagiae/n.t. (4)	Isolation rate [3] Identification [18]
1987	42‡	13‡	30.9‡	Icterohaemorrhagiae/ <i>lai</i> (11) Icterohaemorrhagiae/unidentified (2)	Isolation rate [3] Identification [16]
1996	222	22	9.9	Icterohaemorrhagiae/ <i>lai</i> (22)	

\* Field rodents were collected from middle part of Korea in 1986, 1987. In 1985 and 1996, rodents were collected from southern and middle part of Korea. Rodent species from which *L. interrogans* was isolated was *A. agrarius* except four in 1985.

† Serovar identification was based on the results of cross-agglutinin absorption test, reactivities to monoclonal antibodies and bacterial restriction-endonuclease DNA analysis (BRENDA) method.

‡ Partial data in 1987. Field rodent collected just in September in specific area.

n.t., not tested.

*interrogans*, for generation of these monoclonal antibodies, were done with serovar *copenhageni*, *icterohaemorrhagiae*, *mankarso*, and *ndambari* in serogroup Icterohaemorrhagiae. The reactive patterns of all the isolates were identical to that of serovar *lai* and were

different from those of other 14 serovar reference strains tested (Table 2). Moreover the reactive patterns of strains, HM3 and 18R which were registered as serovar *yeonchon* and *hongchon* [11, 12] were also different from those of isolates. These results

showed that all 22 isolates in this study belonged to serogroup Icterohaemorrhagiae, serovar *lai* of *L. interrogans*.

#### Ecological trend of *L. interrogans* in *A. agrarius*

We compared the results in this study with those of previous surveys (Table 3). In Korea, the numbers of field rodents infected with *L. interrogans* were surveyed in 1984, 1985, 1986 and 1987. The previous results showed that about 15% of field rodents were infected with *L. interrogans* although this varied in 1986 and 1987. These variations were thought to parallel changes in climate and selection of rodent collection sites. All isolates tested (46 cultures) belonged to serogroup Icterohaemorrhagiae and among 42 strains of which serovar was identified, 39 strains were identified as serovar *lai*. It revealed that there had been no significant changes in infection rate of *L. interrogans* in field rodents and distribution of serogroup and serovar.

#### DISCUSSION

Historically the isolation of *L. interrogans* from field rodent in Korea was described by USA military personnel in the 1950s [19, 20]. However, its public health significance has been recognized only recently when laboratory diagnosis and isolation of *L. interrogans* became available in 1984 [3]. In this regard, occurrence of leptospirosis has been under government surveillance as a second class communicable disease since 1987 and nationwide vaccination for high risk groups has been done since 1988 in Korea. Since 1984, several laboratories in Korea have carried out serological diagnosis of leptospirosis in febrile patients. The data from four laboratories including ours showed that more than 10% of febrile patients especially in autumn had leptospirosis [2]. However, since 1988, the incidence of human leptospirosis has fallen rapidly with leptospirosis now being rarely diagnosed, i.e. in less than 1% of febrile patients [2]. The decreased incidence of leptospirosis in Korea has been hard to explain. It is possible that a change of *L. interrogans* ecology could be the cause due to climatic changes, or changes in animal reservoir. Alternatively improved host immunity against *L. interrogans* could be a factor. However, vaccination efficacy has not been evaluated yet in Korea.

A rodent survey has not been carried out since 1988 although isolation of leptospires from man has

continued. The overall detection rate from field rodents was 9.9%. Isolation rate from previous surveys showed about 15%. In 1986, isolation rate was significantly lower, possibly because 1986 was unusually dry-autumn. The high rate in 1987 might have been artificially influenced by selection of capture site where high isolation rates had already been found (Table 3). Thus, it may be that the gross infection rate of *L. interrogans* in field rodents has remained unchanged for the past 13 years. The most common serogroup and serovar was also unchanged. In Korea, during 1984–95, a total of 80 isolates of *L. interrogans* isolated from patients and animals (mostly rodents) were identified. Among these isolates belonging to serogroup Icterohaemorrhagiae, the majority (67 strains) were identified as serovar *lai* and strain HM3 and 18R designated as serovar *yeonchon* and *hongchon* [11, 12], respectively. Two strains which were isolated from man were identified as serogroup Canicola, serovar *canicola* [15]. In this study, strains other than serovar *lai* were not isolated.

In conclusion, there was no significant change of *L. interrogans* in field rodents after vaccination programme initiated. Since this result is only an initial step in the explanation of decreased incidence of human leptospirosis in Korea, the evaluation of host factors like vaccination efficacy should be further studied.

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