NOTE *Bacteriology*

Recovery of Leptospires from Miniature Pigs Experimentally Infected with *Leptospira interrogans* Serovar Manilae Strain UP-MMC under Immunosuppressive Conditions by Dexamethasone

Yoshihiro SAKODA[1)], Michiko NAITO[1)], Mutsumi ITO[1)], Yuki ITO[1)], Norikazu ISODA[1)], Tomohisa TANAKA[2)], Takashi UMEMURA[2)] and Hiroshi KIDA[1,3)]*

[1)] *Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan*
[2)] *Laboratory of Comparative Pathology, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan*
[3)] *Research Center for Zoonosis Control, Hokkaido University, Sapporo 001–0020, Japan*

(Received 30 November 2011/Accepted 25 February 2012/Published online in J-STAGE 9 March 2012)

ABSTRACT. *Leptospira interrogans* serovar Manilae strain UP-MMC was inoculated into miniature pigs to assess its pathogenicity. Leptospires were recovered from the whole blood, kidneys, and livers in the acute phase without showing any clinical signs. Under immunosuppressive conditions by dexamethasone, leptospires were recovered from the kidneys and their genes were detected from the urine in the chronic phase. These results indicate that leptospires persisted in the kidneys until the chronic phase, and excretion of leptospires in the urine was enhanced under immunosuppressive conditions, resulting in horizontal transmission among pigs on farms.
KEY WORDS: experimental infection, immunosuppression, leptospira, swine.

doi: 10.1292/jvms.11-0537; *J. Vet. Med. Sci.* 74(7): 955–958, 2012

Leptospirosis is a zoonosis caused by pathogenic strains of *Leptospira interrogans* [2]. It is known that leptospires are one of the agents of reproductive disorders in domestic animals, especially in pigs. Abortions in pigs associated with leptospirosis have been reported worldwide [1, 3]; however, identification of leptospires on farms is not easy, although the antibodies against leptospires are easily detected since most infections with leptospires in pigs are inapparent in healthy pigs [6, 8]. In the present study, *Leptospira interrogans* serovar Manilae strain UP-MMC was experimentally inoculated into miniature pigs. Clinical signs, replication of leptospires in their blood and organs, and the excretion of leptospires in their urine were investigated under immunosuppressive conditions by dexamethasone to understand the persistence of leptospires in pigs in an animal model using miniature pigs.

Two-month-old female SPF miniature pigs (*Sus scrofa domestica*, NIBS line; Nippon Institute for Biological Science, Yamanashi, Japan) were used for experimental infection. *Leptospira interrogans* serovar Manilae strain UP-MMC, isolated from a human patient with severe leptospirosis in the Philippines [7], was kindly provided by Dr. N. Koizumi, National Institute of Infectious Diseases (Tokyo, Japan). Manilae is one of the common serovars in humans and rats in the Philippines [12, 13], although no case has been reported in pigs. The UP-MMC strain was reported as a highly pathogenic strain to mice and hamsters in experimental infection [7]. This strain was passaged twice in Korthof's medium before animal experiment. Experimental infection was carried out using 4 miniature pigs, and 10^7 UP-MMC strain was inoculated intraperitoneally into each pig. One pig (#1) was euthanized 12 days post-inoculation (dpi) and the other 3 pigs (#2–4) at 37 dpi by pentobarbital (100 mg/kg, intravenous administration). Dexamethasone 0.1 mg/kg (Wako Pure Chemical Industries, Osaka, Japan) was injected intramuscularly into 2 pigs (#3 and #4) every day from 31 to 35 dpi, and phosphate-buffered saline (PBS) was injected intraperitoneally into pig #2 as a control. Body temperature of the pigs was checked every day until 15 dpi and at 22, 29, and 37 dpi. Blood samples were collected from each pig at 0, 3, 6, 9, 12, 15, 22, 29, and 37 dpi. In addition, urine samples were collected from the pigs at 0, 3, 6, 9, 12, 15, 22, 29, 33, 34, 35, 36, and 37 dpi. Sera were also collected for the detection of antibodies against leptospires. At necropsy, tissue samples of the kidneys and livers from each animal were collected aseptically. Isolation, detection of specific genes, and antigen detection of leptospires were also performed for these organs. All animal experiments were carried out in self-contained isolator units (Tokiwa Kagaku, Tokyo, Japan) at the BSL-3 facility of the Graduate School of Veterinary Medicine, Hokkaido University, Japan. The institutional animal care and use committee of the Graduate School of Veterinary Medicine authorized this animal experiment (approval numbers: 18021, and 7095) and all experiments were performed according to the guide-

*CORRESPONDENCE TO: KIDA, H., Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Kita-18 Nishi-9, Sapporo 060–0818, Japan.
e-mail: kida@vetmed.hokudai.ac.jp

Table 1. Detection of antigens and antibodies from miniature pigs inoculated with *Leptospira interrogans* serovar Manilae strain UP-MMC[a)]

Pig ID	Injection	Tested sample	Method	Results of the tests on the dpi												
				0	3	6	9	12	15	22	29	33	34	35	36	37
#1	–	Blood	Recovery	–	+	–	–	–								
		Blood	PCR	–	–	–	–	–								
		Urine	PCR	–	–	–	–	nt								
		Serum	MAT	–	–	–	+	+								
#2	PBS	Blood	Recovery	–	–	–	–	–	–	–	–	nt	nt	nt	nt	–
		Blood	PCR	–	–	–	–	–	–	–	–	nt	nt	nt	nt	–
		Urine	PCR	–	–	–	–	–	–	–	–	–	–	–	–	–
		Serum	MAT	–	–	–	–	+	+	+	+	nt	nt	nt	nt	+
#3	DEX	Blood	Recovery	–	–	+	–	–	–	–	–	nt	nt	nt	nt	–
		Blood	PCR	–	–	+	–	–	–	–	–	nt	nt	nt	nt	–
		Urine	PCR	–	–	–	–	–	–	–	–	+	+	+	+	+
		Serum	MAT	–	–	–	+	+	+	+	+	nt	nt	nt	nt	+
#4	DEX	Blood	Recovery	–	–	–	–	–	–	–	–	nt	nt	nt	nt	–
		Blood	PCR	–	–	–	–	–	–	–	–	nt	nt	nt	nt	–
		Urine	PCR	–	–	–	–	–	–	–	–	–	–	–	–	–
		Serum	MAT	–	–	–	–	+	+	+	+	nt	nt	nt	nt	+

a) After the intraperitoneal inoculation of leptospires into miniature pigs at 0 dpi, pig #1 was euthanized for the detection of leptospires in the acute phase at 12 dpi without injection of immunosuppressant. Dexamethasone (DEX) was injected intramuscularly as immunosuppressant into 2 pigs (#3–4) for 5 days from 31 to 35 dpi. Phosphate-buffered saline (PBS) was injected into pig #2 as negative control according to the schedule of DEX administration. Abbreviations: Recovery: recovery of leptospires in Korthof's medium, MAT: microscopic agglutination test, nt: not tested.

lines of this committee.

In order to isolate leptospires, blood or tissue homogenates were inoculated into 5 m*l* Korthof's medium with 10% normal rabbit serum and incubated for 2 weeks at 30°C. The *flaB* gene of the leptospires was detected by nested PCR from the collected blood, urine, and tissue homogenates. Primer sets reported in Kawabata *et al*. [5] were used for the first amplification according to their protocol. The products of the nested PCR were amplified with flaB-IN-F1: 5′-TTGCTGTGGACAAGACGATG-3′ and flaB-IN-R1: 5′-CCCATATCCGCTCTCTGC-3′ under the condition of 40 cycles of 94 °C; 20 sec, 63°C; 30 sec, and 72°C; 60 sec, from the products of the 1st PCR. Primers and condition for the nested PCR were designed in our laboratory for this experiment. Serum antibodies against challenged UP-MMC strain were detected by the microscopic agglutination test described previously [8]. The samples showing titers of more than 1:100 were considered as positive. The tissues were fixed with 20% phosphate-buffered formalin, sectioned, and stained with hematoxylin and eosin for microscopic examination. For the detection of leptospiral antigen in the tissues, all sections were stained using the streptavidin-biotin immunoperoxidase complex method (Histofine SAB-PO (M) kit; Nichirei Corp., Tokyo, Japan) with a mouse monoclonal antibody against leptospiral lipoprotein LipL32 produced in our laboratory.

None of the pigs inoculated with strain UP-MMC showed any clinical signs or change of their body temperature during the experiments (data not shown). Leptospires were recovered from the blood of 2 pigs (#1 and #3) at 3 and 6 dpi, respectively (Table 1). The *flaB* gene was also detected from the blood of #3 pig at 6 dpi by nested PCR. Although specific antibodies against challenged strain were detected from the sera of all pigs from 9 or12 dpi, no leptospires or their genes were detected from the blood of 2 pigs (#2 and #4) for 37 days in this experiment. Dexamethasone was injected into 2 pigs (#3 and #4) for 5 days from 31 to 35 dpi. Specific genes were detected from the urine of pig #3 every day at 33–37 dpi after the injection of dexamethasone.

Gross and histological lesions were not remarkable in any pigs (data not shown). Recovery of leptospires and detection of the *flaB* gene were performed from the homogenates of the kidneys and livers at 12 dpi (pig #1) and 37 dpi (#2, #3, and #4) (Table 2). Leptospires were recovered from both organs of pig #1 and the kidneys of pig #3. The *flaB* genes were also detected from the kidneys of pig #3. Leptospiral antigens were detected in the lumina of renal tubules and tubular epithelial cells of the kidneys of pig #3 at 37 dpi after the dexamethasone injection (Fig. 1). No leptospires or its genes were detected from any tissues of 2 pigs (#2 and #4).

Leptospires were recovered in the acute phase of infection from the blood of 2 pigs (#1 and #3), and were not detectable after the immunological response against leptospires. In this experiment, leptospiral genes were detected from the urine of pig (#3) in the chronic phase under immunosuppressive conditions by dexamethasone, a synthetic glucocorticoid analogue. Dexamethasone suppresses cell-mediated immunity and inflammatory responses, and enhances the susceptibility and recrudescence of infectious pathogens in the host [4, 10, 11]. The present results indicate that persistent infection of leptospires occurs in pigs and leptospiral replication was reactivated in their kidneys under impaired immunity, resulting in the excretion of leptospires into the urine. Although the morbidity rate of leptospiral infection is usually high on pig farms [8], leptospires have been rarely isolated from pigs on farms in Japan. The present results suggest that most lep-

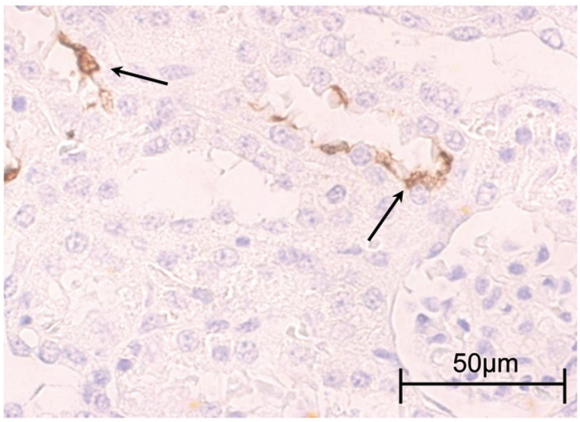

Fig. 1. Detection of leptospiral antigen in the kidneys of miniature pig #3 infected with UP-MMC strain at 37 dpi. After the intraperitoneal inoculation of leptospires into miniature pigs at 0 dpi, dexamethasone was injected intramuscularly into 2 pigs (#3-4) for 5 days at 31 to 35 dpi. Photomicrographs of tissue sections of the kidneys stained immunohistochemically to demonstrate leptospiral antigen LipL32 in the lumina of renal tubules and tubular epithelial cells of pig #3 at 37 dpi (arrows).

tospiral infections are usually inapparent in healthy pigs and impaired immunity due to coinfection with another pathogen and environmental factors leads to the reactivation of leptospiral replication in persistently infected pigs. In addition, a good condition for the replication of leptospires in pigs is to stop giving antibiotics to pigs in the final stage of fattening because of marketing. These excreted leptospires may infect other pigs and infections spread on farms, although most natural infections of leptospires in pigs terminate in the acute phase.

In our preliminary study, the strain for animal experiments and the inoculation route of leptospires were investigated to reproduce the chronic phase and recovery of leptospires under immunosuppressive conditions in miniature pigs (data not shown). As a result, intraperitoneal inoculation of *Leptospira interrogans* serovar Manilae strain UP-MMC led to persistent infection and recovery of leptospires in the chronic phase. In our experiments, specific antigens of leptospires were detected in the lumina of the renal tubule and tubular epithelial cells of the kidneys of an infected pig. The present findings are consistent with the report that leptospiral antigens were diffusely distributed in the interstitium of the kidneys during the acute phase and were localized to the renal tubule during persistent infection in conventional pigs [9]. These data suggest that our experiment using miniature pigs is a good animal model to understand the persistence of leptospires in pigs. Further experiments using miniature pigs are necessary to assess the pathogenicity of leptospires of other serovars, which are recognized as highly susceptible to pigs (e.g. serovars Pomona and Canicola), since no leptospiral infection of pigs with serovar Manilae has been reported.

ACKNOWLEDGMENTS. The authors are grateful to Dr. N. Koizumi, National Institute of Infectious Disease (Tokyo, Japan) for kindly providing the UP-MMC strain. The authors also thank Dr. K. Hirose, Meiji Seika Kaisha, Ltd., (Tokyo, Japan) and Y. Nitta, Okinawa Prefectural Institute of Animal Health (Okinawa, Japan) for their technical support. The present work was supported in part by the J-GRID; the Japan Initiative for Global Research Network on Infectious Diseases and Japan Science and Technology Agency Basic Research Programs.

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Table 2. Detection of antigens from the organs of miniature pigs inoculated with *Leptospira interrogans* serovar Manilae strain UP-MMC[a)]

Pig ID	Injection	Dpi	Kidneys			Livers		
			Recovery	PCR	IHC	Recovery	PCR	IHC
#1	-	12	+	–	–	+	–	–
#2	PBS	37	–	–	–	–	–	–
#3	DEX	37	+	+	+	–	–	–
#4	DEX	37	–	–	–	–	–	–

a) After the intraperitoneal inoculation of leptospires into miniature pigs at 0 dpi, pig #1 was euthanized for the detection of leptospires at 12 dpi without injection of immunosuppressant. Phosphate-buffered saline (PBS) or dexamethasone (DEX) was injected intramuscularly into 3 pigs (#2-4) for 5 days from 31 to 35 dpi as explained in Table 1. Abbreviations: Recovery: recovery of leptospires in Korthof's medium, IHC: immunohistochemical staining.

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