Overseas Practice on（Field Epidemiology・Collaborative Research）
report form（For Student）

2014/07/31 (Year/Month/Day)

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Year (Grade)	2nd year PhD student
Place of practice	Philippine Carabao Center National Headquarters and Genepool (PCC) Science City of Muñoz, Nueva Ecija Philippines 3120 College of Public Health, University of the Philippines-Manila (CPH-UPM) 625 Pedro Gil Street, Ermita Manila, Philippines
Period of practice	May 25 – June 29, 2014
Purpose	Field epidemiology on understanding the circulation of *Leptospira* among water buffaloes in an intensive farm setting

Leptospirosis is considered as one of the most widespread and significant zoonotic diseases in the world. Our previous study investigated the water buffalo, an indispensable animal in the Philippines that may be a source of leptospirosis in humans. Results revealed that tested sera showed high reactivity to recombinant outer membrane protein (rOMP) in ELISA while half of the urine samples were positive for Lepto-*rrs* LAMP. Furthermore, sequencing of nested PCR products revealed the presence of *L. kirschneri* and *L. borgpetersenii* which are both pathogenic to humans. These results showed that the *Leptospira* infection seems to be well maintained in this animal, especially in pure confinement setting, which alarm for public health concern especially for farmers, veterinarians and farm workers who are in close contact with this animal. However, acquisition and circulation of pathogenic *Leptospira* sp. within the herd is still unclear, hence, this follow-up study was conducted.

The objectives of this study are to identify the age and location where the water buffalo acquire *Leptospira* in an intensive farm setting using serological and molecular detection tests. We would also like to determine the prevailing serogroups present in water buffalo herd interacting within the farm. And lastly, to evaluate the sensitivity and specificity of the developed indirect ELISA system for leptospirosis prevalence determination compared with reference method which in microscopic agglutination test (MAT).

Optimization of the developed ELISA system. At first, optimization of ELISA system was performed for buffalo samples in the Philippines since previous optimization was done using samples from Japan, where antibody titer may be lower as compared to Philippines' samples. Aside from sample dilution, almost all on the ELISA steps were further optimized for the purposes of higher sensitivity and specificity, saving of expensive reagents and ease of using this test. After series of testing, we finally came up with the ELISA condition of (1) using LipL32B (*L. borgpetersenii*) as coating antigen, (2) 1% BSA for blocking, (3) 400X and 2-step dilution of serum sample, (4) overnight incubation of antigen-antibody interaction at 4°C and (5) 2000X dilution of

HRP-labeled detection antibody.

Presentation of study proposal at the College of Public Health-University of the Philippines-Manila (CPH-UPM). One of the important parts of this study is the identification of predominant serogroups circulating in water buffaloes using MAT which is the reference test. This can only be performed at CPH-UPM as they have the laboratory facility that kept a battery of *Leptospira* serovars that represents those that are present in the Philippines. We presented our research proposal for a collaborative research (Picture 1) and it was a success. We exchanged ideas on how to improve the study more and we agreed on providing technical skills and resources for each party.

Sample collection. Serum (n=170) and urine (n=164) samples were collected from different age groups and locations in one buffalo farm. Figure 1 and Table 1 showed the lay-out of the farm and the number of samples collected per age group/ location, respectively. Blood collection on calf and adult buffalo were shown in Pictures 2 and 3, respectively. After collection, serum was collected (Picture 4), labeled properly and stored at -80°C until used in the study. Urine samples, on the other hand, were processed within the day after collection for DNA extraction using centrifugation and boiling method, and then stored at -30°C until used in the study.

ELISA. The optimized ELISA condition was applied in testing the water buffalo sera (Picture 5). Figure 2 showed the ELISA result obtained from different age groups/ location of buffaloes in the farm. Grouping of animals in a pen may play an important role in the acquisition of *Leptospira*, as shown in the result from grouped animals compared with calves which are kept in individual pen. Variability of ELISA results were observed from the grouped animal which may suggest intermittent infection. However, these findings will not be considered concrete evidence of *Leptospira* infection as of this moment due to high non-specific reaction when comparing the reactivity of produced rLipL32 and negative control antigen in a separate experiment. Further purification of the produced recombinant antigen is necessary or use of other kinds of tag in antigen is needed to confirm the reliability of the result.

MAT. The MAT test is comprised of two steps, (1) screening and (2) quantitative MAT. For the MAT screening, each serum was reacted with the 39 panel of antigens in a sterile, flat-bottom 96 well plate, with a final serum dilution of 1/20. The plate was mixed and incubated for 2 hours at 30°C. After incubation, agglutination was observed column by column using dark field microscope (Picture 6). Every serum which gives an agglutination of $\geq$50% of the leptospires (as compared with control antigen) is considered positive (Picture 7). A quantitative MAT, on the other hand is carried out by making two-fold serial dilutions of the serum to determine the antibody titer for each of the positive antigens. However, this step was not performed during the visit because of being laborious and time consuming of the test starting from the MAT screening.

Samples that have high OD values in ELISA were first subjected for MAT screening, and the result was shown on the left column of Table 2. At the final serum dilution of 1/20, tested samples agglutinate more than one serovar in the MAT panel of antigens. It somehow correlates with the ELISA result where the high OD

values agglutinate many serovars. *L. borgpetersenii* serovars Mini and Serjoe and *L. interrogans* serovar Hebdomadis were the most commonly agglutinated serovars by buffalo sera, where *L. borgpetersenii* is considered a species specifically harbored by ruminants. However these data should be further confirmed by Quantitative MAT to clarify the actual serovars infecting the water buffaloes.

***lipL32* nested PCR.** Amplification of *lipL32* gene means presence of pathogenic *Leptospira* sp. from urine of water buffaloes. Table 3 showed the nested PCR result of 164 urine samples from water buffaloes. Results from nested PCR correlates well with the ELISA result since the positive samples were detected only from grouped animals. Sequence analysis of the nested PCR products is now ongoing for *Leptospira* species identification.

In summary, introduction of an animal in a group pen/ house in an intensive farming system may be critical in contracting leptospirosis and eventually, intermittent infection, as shown from serological and molecular tests. There seem to be a correlation between ELISA and MAT results; however, eliminating the problems on the non-specific reaction in ELISA is important to confirm the reliability of results. Continuation of MAT test is also important as a reference method for the developed ELISA system. Finally, this information may serve as a model in concentrating the critical age of animal and areas in the farm where *Leptospira* is acquired and maintained, thus, appropriate control measures can be done.

（Field Epidemiology・Collaborative Research） Evaluation by supervisor

Institution・Official title・Name	Hokkaido University Research Center for Zoonosis Control・ Professor ・Yasuhiko SUZUKI 印
Describe overall evaluation on the applicant's activity in overseas practice. Comprehensive study on the prevalence of leptospira infection in water buffaloes was performed by using ELISA, MAT and PCR technology. Results clearly showed that introduction of an animal into a group pen/ house to be a cause of leptospira infection and suggested the need of counter measure focus on this. Hence, Mr. Villanueva's collaborative research with the College of Public Health-University of the Philippines-Manila and Philippine carabao center seemed to be success. However, the continuation of MAT is recommended to identify the serovars of leptospira infecting water buffaloes. In his future study	

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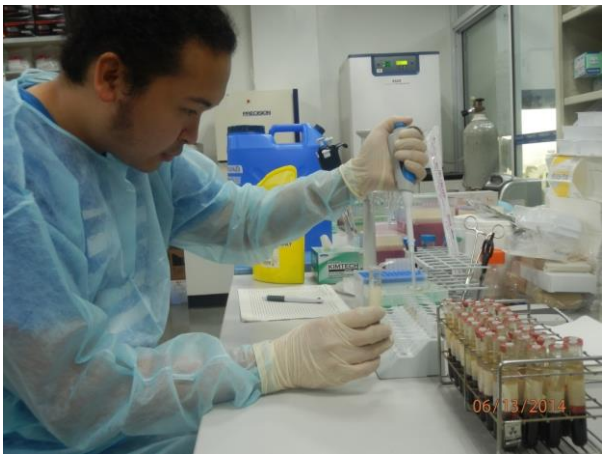
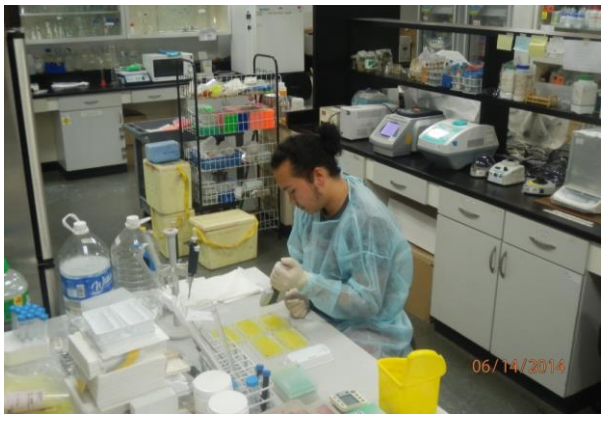
Picture 1. Meeting with Professor Yasutake Yanagihara (far left)and Dr. Nina Gloriani (third from the left) about the proposed study on leptospirosis.

Picture 2. Blood collection on water buffalo calf from one intensive farm in the Philippines

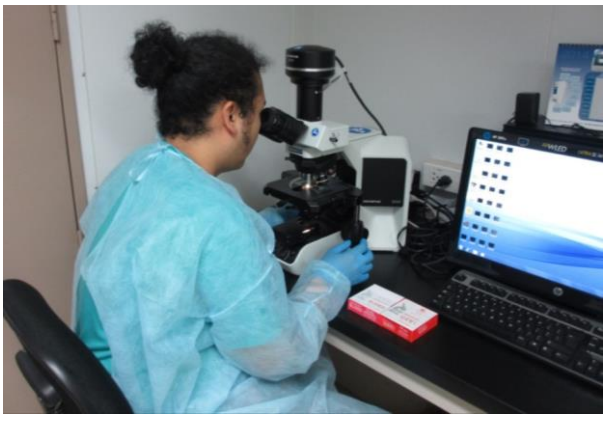
Picture 3. Blood collection on adult water buffalo from one intensive farm in the Philippines



Picture 4. Preparation of samples to be used in the study

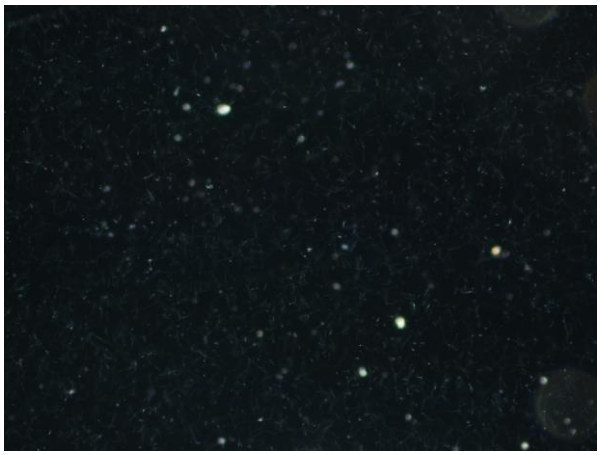


Picture 5. Performing of ELISA test



Picture 6. MAT screening test using dark field microscope.

Picture 7. Positive reaction (>50% agglutination) of water buffalo serum to *L. interrogans* serovar Manilae during the MAT screening.



Picture 8. Negative reaction because of the presence of free *L. interrogans* serovar Manilae after reacting with water buffalo serum.

Picture 9. Discussion of MAT result with the staffs of CPH-UPM and Japanese experts.

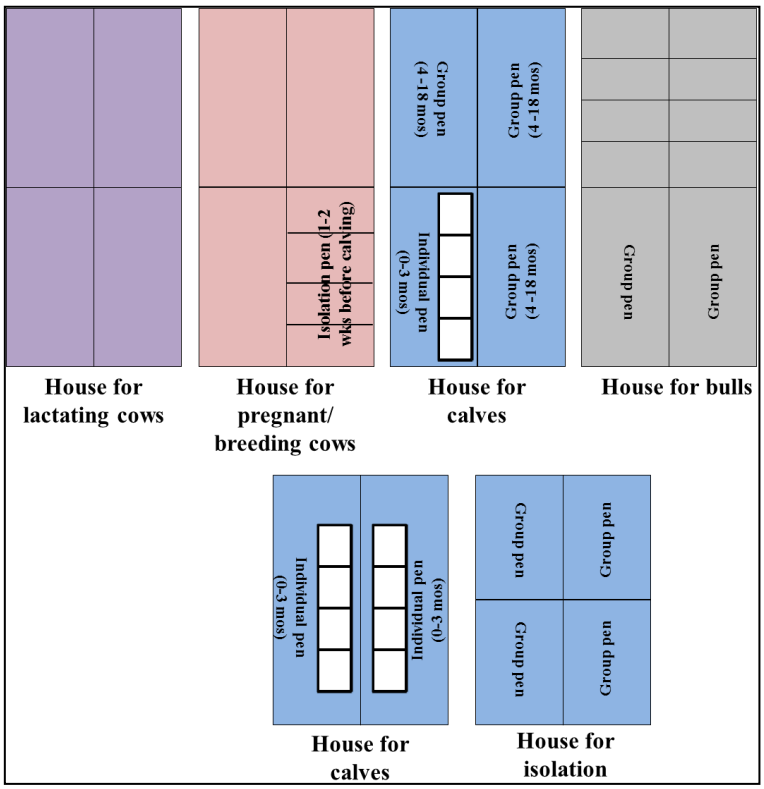


Figure 1. Lay-out of the farm where the samples are collected

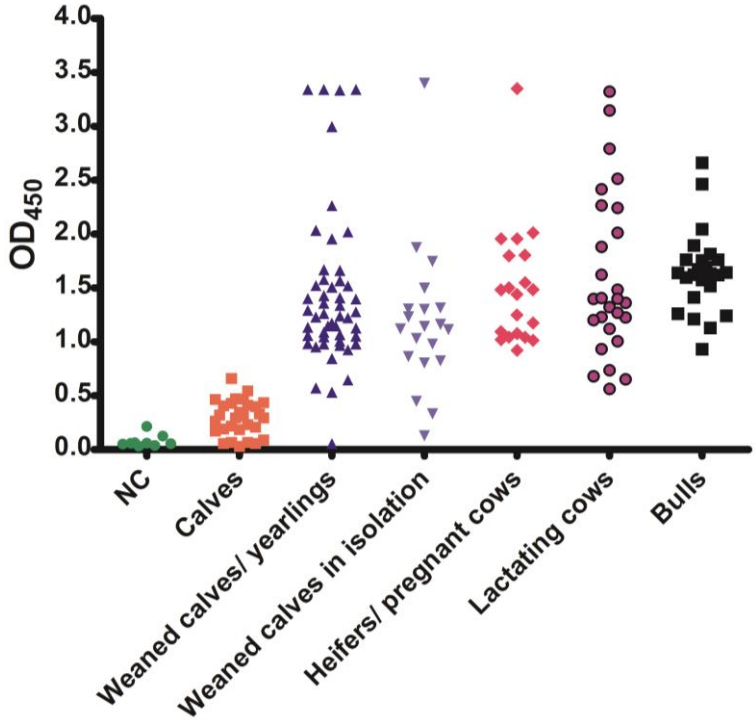


Figure 2. ELISA result of the water buffalo samples

Table 1. Water buffalo serum samples collected in the farm.

House/ pen	No. of samples collected
House for calves	
Pre-colostral	8
0-14 days	5
15-30 days	2
1-2 months	2
2-3 months	12
House for weaned calves/ yearlings	50
House for weaned calved in isolation	20
House for heifers/ pregnant cows	20
House for lactating cows	27
House for bulls	24
Total	**170**

Table 3. *lipL32* nested PCR result from water buffalo urine samples.

House/ pen	No. of samples tested	*lipL32* nested PCR result
House for calves		
Pre-colostral	8	0
0-14 days	5	0
15-30 days	2	0
1-2 months	2	0
2-3 months	12	0
House for weaned calves/ yearlings	50	17 (34%)
House for weaned calved in isolation	20	1 (5%)
House for heifers/ pregnant cows	20	11 (55%)
House for lactating cows	21	6 (29%)
House for bulls	24	13 (54%)
Total	**164**	

Table 2. The 39 *Leptospira* panel of antigens used for MAT and the result of MAT screening.

		Microscopic Agglutination Test (MAT) panel of antigens					
	No. of samples with agglutinating antibody (MAT screening)	**Genus and genomospecies**	**Serogroup**	**Serovar**	**Strain**	**Country of origin**	**Source**
A1	8	*L. borgpetersenii*	Tarassovi	Tarassovi	Perepelitsin	USSR	human
A2	15	*L.interrogans*	Pyrogenes	Manilae	LT 398	Philippines	rat
A3	5	*L.interrogans*	Canicola	Canicola	Hond Utrecht IV	Netherlands	dog
A4	21	*L. borgpetersenii*	Javanica	Poi	Poi	Italy	human
A5	14	*L.interrogans*	Pyrogenes	Pyrogenes	Salinem	Indonesia	human
A6	28	*L. kirschneri*	Grippotyphosa	Grippotyphosa	Moskva V	USSR	human
A7	23	*L.interrogans*	Autumnalis	Autumnalis	Akiyami A	Japan	human
A8	6	*L.interrogans*	Icterohaemorrhagiae	Icterohaemorrhagiae	RGA	Belgium	human
A9	29	*L.interrogans*	Icterohaemorrhagiae	Copenhageni	M20	Denmark	human
A10	5	*L.interrogans*	Bataviae	Los banos	LT101-69	Philippines	rat
A11	13	*L. kirschneri*	Grippotyphosa	Ratnapura	UP-BL-FR13	Philippines	rat
A12	27	*L.interrogans*	Hebdomadis	Hebdomadis	Akiyami B	Japan	human
A13	9	*L.interrogans*	Australis	Australis	Akiyami C	Japan	human
A14	20	*L. biflexa*	Semaranga	Patoc	Patoc I	Italy	water
A15	4	*L.interrogans*	Icterohaemorrhagiae	Icterohaemorrhagiae	Ictero No. 1	Japan	human
A16	10	*L. meyeri*	Semaranga	Semaranga	Veldrat Semarang 173	Indonesia	rat
A17	28	*L.interrogans*	Pomona	Pomona	Pomona	Australia	human
A18	27	*L. interrogans*	Serjoe	Hardjo	Hardjoprajitno	Indonesia	human
A19	14	*L. interrogans*	Australis	Australis	Ballico	Australia	human
A20	29	*L. fainei*	?	Hurstbridge	BUT 6T	Australia	pig
A21	10	*L. biflexa*	Andamana	Andamana	CH 11	Andaman Islands	human
A22	3	*L. noguchii*	Panama	Panama	CZ 214 K	Panama	opossum
A23	6	*L. interrogans*	Djasiman	Djasiman	Djasiman	Indonesia	human
A24	8	*L. borgpetersenii*	Autumnalis	Ballum	Fort Bragg	USA	human
A25	30	*L. interrogans*	Hebdomadis	Hebdomadis	Hebdomadis	Japan	human
A26	15	*L. interrogans*	Grippotyphosa	Grippotyphosa	K5	Philippines	rat
A27	8	*L. borgpetersenii*	Javanica	Javanica	K6	Philippines	rat
A28	13	*L. interrogans*	Bataviae	Los banos	K37	Philippines	rat
A29	12	*L. interrogans*	Pyrogenes	Manilae	K64	Philippines	rat
A30	0	*L. alexanderi*	Manhao	Manhao 3	L60	China	?
A31	29	*L. noguchii*	Louisiana	Louisiana	LSU 1945	USA	armadillo
A32	8	*L. borgpetersenii*	Celledoni	Anhoa	LT 90-68	Vietnam	human
A33	33	*L. borgpetersenii*	Serjoe	Serjoe	M 84	Denmark	mouse
A34	20	*L. yanagawae*	Semaranga	Sao Paolo	Sao Paolo	Brazil	water
A35	38	*L. borgpetersenii*	Mini	Mini	Sari	Italy	human
A36	10	*L. weilii*	Sarmin	Sarmin	Sarmin	Indonesia	human
A37	20	*L. borgpetersenii*	Javanica	Javanica	Veldrat Batavia 46	Indonesia	rat
A38	10	*L. santarosai*	Shermani	Shermani	1342 K	Panama	spiny rat
A39	13	*L. kirschneri*	Cynopteri	Cynopteri	3522 C	Indonesia	bat