F-062 Investigation on the route of transmission and infectious risk of West Nile Virus and blood parasites by migratory birds (Abstract of the Final Report)

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[Abstract]

The epidemiological survey was conducted on West Nile Virus in migratory shorebirds and endangered avian species in Japan such as Blakiston's fish owl, White-tailed eagle, Japanese crane and Okinawa rail and to evaluate the outbreak possibility of WNV. We also conducted assessing the invasion risk of blood parasite such as avian malaria (*Plasmodium* spp. / *Haemoproteus* spp.). Because these infectious diseases may cause mass death on Japanese wild birds. In addition to WNV and avian malaria monitoring, we assessed the invasion risk of other highly pathogenic agent such as parasitic helminthes and protozoans in resident birds and wild mammals. Because they are distributing in same area with endangered avian species and highly pathogenic agents may be transmitted between migratory shorebirds and resident birds and mammals.

There were no antigen and gene positive cases of WNV in 1,131 wild birds in Japan, 16 birds in Russia and 42 birds in Thailand. However 52 serum samples from ducks had N-antibodies against Nakayama (JEV), Beijing-1 (JEV) and Eg-101(WNV). More than 25 strains of avian malaria were detected from the migrating and resident birds captured in Okinawa and mainland Japan. The sequences of mtDNA cytochrome *b* partial gene of avian malaria from the waders captured at Kushiro Wetland and the periphery of Komuke Lake in Hokkaido was completely same with the one from Japanese cranes (*Grus japonensis*) inhabit Kushiro Wetlands. This indicates that the transmission of vector borne diseases between migratory birds and native birds could occur at wetlands somewhere in Japan. Three different genera of parasites (nematode, Acanthocephala and trematode) were found in 46 intestinal samples of Okinawa rail. And we successfully established nested PCR and duplex real-time PCR to identify those parasites.

1. Introduction

The infection of the West Nile Virus (WNV) was found in waders/sandpipers and some other bird species in Western Siberia and Alaska. This suggested that a mass death on Japanese wild birds would occur if the migrating birds came from these areas to wetlands and mud lands in Japan with the agent. In addition to WNV, there were some pathogenic agents causing mass death such as blood parasite. These pathogenic agents are considered as one of the risk factors for the extinction on avian species in Japan. However there is no monitoring system for highly pathogenic agent. Therefore, we plan to perform an epidemiological survey of WNV, blood parasite as well as other pathogenic agents in migratory shorebirds and other wildlife in Japan.

2. Research Objective

The epidemiological survey was conducted on West Nile Virus in migratory shorebirds and endangered avian species in Japan such as Blakiston's fish owl, White-tailed eagle, Japanese crane and Okinawa rail to evaluate the outbreak possibility of WNV. We also conducted assessing the invasion risk of blood parasite such as avian malaria (*Plasmodium* spp. / *Haemoproteus* spp.). Because these infectious diseases may cause mass death on Japanese wild birds. In addition to WNV and avian malaria monitoring, we assessed the invasion risk of other highly pathogenic agent such as parasitic helminthes and protozoans in resident birds and wild mammals. Because they are distributing in same area with endangered avian species, highly pathogenic agents may be transmitted between migratory shorebirds and resident birds and mammals. The goal of this study is not only to maintain an ecosystem health, but also to do human/animal health.

3. Research Method

(1) Study on the infection disease in migrating birds

WNV monitoring was conducted in Hokkaido during south-migration period and in Okinawa and Kumamoto during north-migration period from 2006 to 2008. Oral swabs for WNV diagnosis were collected from 1,131 birds of 46 species. All swab samples were used for WNV diagnosis by VecTest and LAMP method. We conducted WNV monitoring at Bolon nature reserve in Russia and Kasetsart university campus in Thailand as well. In Bolon nature reserve, 16 chicks of Oriental white stork (*Ciconia boyciana*) were captured and 42 wild birds were captured in Kasetsart University. Oral swabs were collected from all wild birds to diagnosis WNV infection by VecTest.

(2) Study on monitoring for blood parasite

Infection of several avian blood parasites; avian malaria (*Plasmodium* spp./*Haemoproteus* spp.), *Leucocytozoon* spp., *Trypanosoma* spp, and microfilaria, and avian pox virus among waders and Japanese wild birds including endangered species were clarified at wetlands or mudland on the migrating routes of waders; Hokkaido, Kanto district, Okinawa Main Island, Ishigaki Island, South Borodino Island. The study was also carried out at Thailand and Russia. Furthermore, blood sucking insects, mainly mosquitoes, were collected at the same places, identified as species level and examined molecular biologically if they reserve avian blood parasites or virus genes. The genes of those pathogenic microorganisms from birds and mosquitoes were amplified by PCR and

analyzed as the bio-indicator of WNV because the life cycle of these microorganisms are transmitted by arthropod vectors as same as WNV.

(3) A study on the risk assessment for transition of highly pathogenic agents between migratory shorebirds and resident birds and mammals in Japan.

General screening survey: Tracheal swab materials of the present general survey were derived from 870 individuals were examined using the WNV kit. If false positive reaction was obtained, the real-time polymerase chain reaction (PCR) for the RNA extracted from brain, heart and kidney of an avian individual, and cell culture methods were also applied for a definitive diagnosis. Seroepidemiologic study for flavivirus infections: Serum samples from 92 wild ducks captured and 10 captive ducks kept in central part of Hokkaido, were subjected to seroepidemiologic and virologic study for flavivirus infections. Twenty spotbills (*Anas poecilohyncha*), 50 mallards (*Anas platyhynchos*), 16 northern pintails (*Anas acuta*), and 6 wigeon (*Anas penelope*) were captured on October and November, in 2005 and 2006. Neutralization (N) test were conducted by 50 % focus reduction method using 2 JEV strains (Nakayama and Beijing-1).

(4) Development of nested PCR assay for molecular diagnosis of helminth in Okinawa rail (*Gallirallus okinawae*).

Three different genera of parasites (nematode, Acanthocephala and trematode) were found in 46 intestinal samples of Okinawa rail, in which nematodes obtained from 26 samples were identified as genus *Heterakis* by morphological observation, and another 2 samples were found to be infected with Acanthcephala. Trematode were isolated from 13 samples and classified as Plagiorchiida. Species specific PCR primers and fluorescent labeled probe were designed for nematode and trematode based on cytochrome C oxidase subunit 1 (COX1) gene at mtDNA loci to conducted nested PCR and duplex probe-based real-time PCR.

4. Result and Discussion

(1) Study on the infection disease in migrating birds

The result is that all samples collected in Hokkaido and Okinawa showed negative results. This result suggested that expanding distribution of WNV into Japan by migratory shorebirds has not yet occurred so far. And all samples collected in Bolon nature reserave, Russia and in Kasesart University, Thailand showed negative except one samples collected from White-thorated kingfisher (*Halcyon smyrnensis*). The sample was tested again using RT-PCR and LAMP method. The both result showed negative. Although we were not able to detect WNV in Russia and Thailand, according to previous WNV monitoring result in Bolon nature reserve, Russia, some WNV cases were detected in the reserve. Therefore we should continue WNV monitoring in Hokkaido to detect WNV expansion in early stage.

(2) Study on monitoring for blood parasite

Avian malaria infection was found in waders captured at Kushiro Wetland and the periphery of Komuke Lake, Hokkaido. The sequences of mtDNA cytochrome *b* partial gene of avian malaria from the waders were completely same with the one from Japanese cranes (*Grus japonensis*) inhabit Kushiro Wetlands. This indicates that the transmission of vector borne diseases between

migratory birds and native birds could occur at wetlands somewhere in Japan. Results of this study indicated that the host-switching of vector borne diseases could occur between migrating waders and native birds which inhabit sympatric at wetlands and/or mudlands. Research on mosquitoes inhabits wetland and their breeding season indicated that most species probably transmit WNV. The peak breeding season of mosquitoes at wetlands in Kanto district and the main land Okinawa was overlapped with the peak of migratory season of waders and the partial gene of avian malaria was detected from several mosquitoes at the period. This means that the vector borne diseases could spread among wild birds at wetlands when waders carrying the vector borne diseases flied from Siberia or Alaska.

(3) A study on the risk assessment for transition of highly pathogenic agents between migratory shorebirds and resident birds and mammals in Japan.

1) General screening survey: Among the all materials examined, 868 individuals showed negative reaction of the kit. Two mountain sparrows (order Passeriformes), nevertheless, showed false-positive reaction. Finally, both the real-time polymerase chain reaction (PCR) and the virological methods with the materials derived from the sparrows were done, and each definitive negative reaction was confirmed. All survey was performed in the Wild Animal Medical Center (WAMC) (http://www.google.co.jp/search?hl=ja&q=wamc) under the P2 level, Rakuno Gakuen University, to prevent the so-called pathogen spill over to human, captive animal or nature. Although such false-positive reaction is not rare because it has been reported that false-positive results occurred with the materials derived from the order Passeriformes, a further examination should be halted before the confirmation with true negative reaction. In the present situation, the application of the kit for the monitoring of WNV infection in the Japanese avian species seems to be valuable for screening (first step level), and we could recommend its usage in an avian research institute, field center and so on. However, before the usage, a responsible body (or director, president, dean etc) managing such institute or center must have a definitive (negative/positive) confirmation system between the institute and virological researchers (or institute) to prevent public panic. After the confirmation of each negative result, each body was dissected for further virological (including influenza virus), pathological, toxicological and parasitological examinations, because protozoan, helminth and/or arthropod parasites can become highly pathogenic agents for wild avian species. Two cases of prominent helmithological studies performed between 2006 and 2008: As one of such helminthological studies, patial epidemiological analysis of parasitic helminths obtained from Anseriformes birds were conducted by K function method with 417 individuals of the birds collected in Hokkaido, Japan. All analysis was carried out using spatial analyzing module, S+SpatialStats. Recently, the spatial epidemiological analysis of infectious agents have been performed in human medicine, but there is no trial of the wildlife medical field (Yoshino et al., 2008a).

And, we have on-going studies about endangered species. The Japanese rock ptarmigan (*Lagopus mutus japonicus*) and the Okinawa rail (*Gallirallus okinawae*) are endangered avian species in Japan, and both have been given the status of National Natural Treasures by the Japanese Government. Despite these efforts, the pathogenic or parasitic agents of this rail species have

rarely been described, and especially, no parasitic nematodes of these rails have so far been recorded. In the risk assessment process, it is thus essential to determine the parasitic agents of the species. And, we have reported, for the first time, on the genus *Heterakis* (Nematoda: Heterakidae) obtained from both avian species. In February, 2002, two rock ptarmigans were captured in Mt. Tateyama, Japan, and the birds had been kept in Ueno Zoological Garden, Tokyo, from then to 2003. However, these ptarmigans died due to an unknown cause (probably, non-infectious diseases), and a post-mortem examination was performed. During the examination, one male and one female in total of the parasitic nematode genus *Heterakis* were obtained from the large intestinal tract of one individual of the two examined. From 15 May to 26 June 2004, six dead rails seemingly killed in traffic accidents in the northern part of Okinawa Island, Japan, were taken to the Laboratory of Intellectual Fundamentals for Environmental Studies, the National Institute of Environmental Studies, Japan, for inclusion in the Environmental Specimen Time Capsule project (www.nies.go.jp/index-j.html). The carcasses were stored at +4℃ until post-mortem examination. During the examination, a total of nine individual parasitic nematodes of the genus *Heterakis* were obtained from the large intestinal tracts of three of the rails examined. Between two and five nematodes were counted in each rail. The nematode specimens from the ptarmigan (specimen No.: WAMC-As-8864-1) and from the rails (specimen Nos.: NIES-15A-1, 16A-1, 19A-1) have been deposited in the Wild Animal Medical Center, Rakuno Gakuen University, and in the Laboratory of Intellectual Fundamentals for Environmental Studies, the National Institute for Environmental Studies, Japan, respectively. Although only one male was obtained from the ptarmigan, it was identified as the cosmopolitan species *H. gallinarum*, because of the spicule length (right spicule = 1.647 mm, left one = 0.475 mm) and arrangement of the caudal papillae of the male individual present. On the other hand, the species from the rails is different from *H. gallinarum*, because of the presence of two equal spicules. According to taxonomical reviews and/or keys of the genus *Heterakis*, there is only one known species of *Heterakis* with two equal or nearly equal spicules between 1.3 and 2.3 mm in length of the present specimens , namely *H. isolonche* [Synonyms: *H. lanei, H. neoplastica*; Host genera: *Crossopt, Gennaeus, Ithagenes, Lophophorus, Lophura, Phasianus, Polyplectron, Thaumalea*; Localities (including zoological gardens): Europe, India, China, USA], and the nematodes from the rails belonging to this species. This result has been supported by molecular analyses by Zhao et al. (in press). *H. gallinarum* inhabits the poultry's caecum and is important as the transport host for the highly pathogenic protozoan *Histomonas meleagridis* that causes an often-fatal disease for many birds, blackhead disease. Moreover, larvae of *H. gallinarum* can cause typhlitis with severe diarrhea, weight loss and depression with lymphocytic infiltration and granuloma formation by invading the intestinal wall of infected. Hence, the nematodes of the genus *Heterakis* should be taken into epidemiological consideration, although no pathogenicities regarding *H. isolonche* have so far been reported.

2) Seroepidemiologic study for flavivirus infections of wild and captive ducks: Diseases caused by flaviviruses such as Japanese encephalitis virus (JEV) and West Nile virus (WNV) are recognized as emerging and re-emerging diseases. Recently, distribution of JEV genotype 1 and WNV

expanded to new territories. JEV genotype 1 was distributed from northeast Thailand to Cambodia before 1990s and recently isolated from Vietnam, China, Republic of Korea, Japan and Australia. WNV was distributed over Africa, Europe, Middle-east, Central and Western Asia; however WNV emerged in 1999 and established in North America and continue to be great threat to public health there. And around the same time, WNV were detected in Siberia including far-east Russia. Invasion of WNV to Japan became one of the big concerns. The mechanisms of expanded distribution of these flaviviruses were unknown, although bird migration is suggested to be involved in this matter. Among 92 wild ducks examined, spotbill is considered as summer bird (partly resident and wondering bird), mallard is resident (partly winter and wondering bird), northern pintail and wigeon are winter birds in Hokkaido, and 52 serum samples from ducks were subjected to virus isolation with negative results. As result of the neutralization test using Nakayama, Beijing-1, and WNV Eg-101 strains, 85.9%, 97.8%, 66.3% of serum samples had N-antibodies against Nakayama (JEV), Beijing-1 (JEV) and Eg-101(WNV) respectively. Each geometric mean titer (GMT) was 1.36, 1.45 and 1.11, respectively. These results indicate most of wild ducks in this study have been exposed by flavivirus. Spotbill (summer bird) had highest GMT against both strain of JEV among 4 species of ducks. While winter birds, northern pintail and wigeon, had higher GMT against WNV than spotbill and mallard. These results with recognized migration routes of wild ducks suggested summer birds, spotbills, were infected by JEV, while winter birds, northern pintail and wigeon, were infected by WNV. On the other hands, highly positive reaction in the captive was obtained as well, but because this survey is on-going project, final conclusions should be waited until further investigation.

(4) Development of nested PCR assay for molecular diagnosis of helminth in Okinawa rail (*Gallirallus okinawae*)

A fresh stool sample containing both nematode and trematode eggs was tested by the nested PCR assay, and the results showed the specific primers were good enough for discrimination of parasites. Besides, we attempted to develop duplex nested PCR assay for rapid and successful identification and differentiation of those parasites in Okinawa rail. These data suggest that this new nested PCR protocol is highly effective in distinguishing the species of parasites of Okinawa rail, which could be applied in clinical detection in the future.

To further improve the sensitivity and specificity of molecular diagnostic method in the identification of parasites in Okinawa rail, a duplex probe-based real-time PCR assay was developed. Forty two fecal samples were applied to validate and compare the effectiveness between nested and duplex real-time PCR. The result showed both the two molecular diagnostic methods can detect and identify the parasitic eggs in feces. However the duplex real-time PCR assay manifested not only higher detection sensitivity for trematode eggs but also a faster and simpler detection procedure, and thus it is of more practical value in clinical diagnosis.

Major Publications

1) Sato, Y., Hagihara, M., Yamaguchi, T., Yukawa, M. and Murata, K. 2007. Phylogenetic comparison of *Leucocytozoon* spp. from wild birds of Japan. J. Vet. Med. Sci, 69:1,55-59.

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4) Murata, K., Tamada, A., Ichikawa, Y., Hagihara, M., Sato, Y., Nakamura, H., Nakamura, M., Sakanakura T. and Asakawa M. 2007. Geographical Distribution and Seasonality of the Prevalence of *Leucocytozoon lovati* in Japanese Rock Ptarmigans (*Lagopus mutus japonicus*) found in the Alpine Regions of Japan. J. Vet. Med. Sci. 62: 171-176.

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11) Yoshino, T., Onuma, M., Nagamine, T,. Inaba, M., Kawashima, T., Murata, K., Kawakami, K., Kuwana, T. and Asakawa, M. 2008. First record of the genus Heterakis(Nematoda: Heterakidae) obtained from two scarce avian species, Japaneserock ptarmigan (*Lagopus mutus japonicus*) and Okinawa rails (*Gallirallus okinawae*),in Japan. Jpn. J. Nematol., 38 (2), 89-92.

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13) Zhao, C., Onuma, M., Asakawa, M. and Kuwana, T. 2009. Preliminary studies on developing a nested PCR assay for molecular diagnosis and identification of nematode (*Heterakis isolonche*) and trematode (*Glaphyrostomum* sp.) in Okinawa rail (*Gallirallus okinawae*). Vet Parasitol. in press.

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