Control of emerging infectious diseases between free-ranging wildlife and captive animals in a zoo by diagnostic methods, epidemiological surveillances and biosecurity measures

（動物園における野生動物と飼育動物の新興感染症の診断、疫学調査およびバイオセキュリティ対策による制御）

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ABBREVIATION

ABTS: 2,2'-azino-bis (3-etylbenzthiazoline-6-sulfonic acid) diammonium salt

AIV: avian influenza virus

AIDS: acquired immunodeficiency syndrome

APV: avipoxvirus

BCIP: 5-bromo-4-chloro-3-indolylphosphate

BLAST: basic local alignment search tool

Cc: Carrion Crow (*Corvus corone*)

Cm: Large-billed Crow (*Corvus macrorhynchos*)

CLSI: Clinical and Laboratory Standards Institute

DDBJ: deoxyribonucleic acid data bank of Japan

DNA: deoxyribonucleic acid

DT: definitive phage type

EID: emerging infectious disease

ELISA: enzyme-linked immunosorbent assay

FRAME: Replacement of Animals in Medical Experiments

HIV: human immunodeficiency virus

HPAI: highly pathogenic avian influenza

HPAIV: highly pathogenic avian influenza virus

HRP: horseradish peroxidase

IgG: immunoglobulin G

IgM: immunoglobulin M

ITS: internal transcribed spacer

NBT: nitrotetrazolium blue

OD: optical density

PBS: phosphate buffered saline

PBS-T: PBS containing 0.05% Tween 20

PCR: polymerase chain reaction

PFGE: pulsed-field gel electrophoresis

P4b: 4b core protein

RNA: ribonucleic acid

SD: standard deviation

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

S. Typhimurium: *Salmonella enterica* subspecies *enterica* serovar Typhimurium

UK: The United Kingdom of Great Britain and Northern Ireland

USA: The United States of America

WAZA: World Association of Zoos and Aquariums

WB: western blot assay

WHO: World Health Organization

WNV: West Nile virus

CONTENTS

PREFACE

Occurrence of infectious diseases in wildlife can be a natural phenomenon; however, there is an increasing trend of anthropogenically driven novel or introduced diseases occurring worldwide (Daszak et al. 2000; 2001). Wildlife emerging infectious diseases (EIDs) caused by introduced novel pathogens can have severe negative impacts on host populations, leading to catastrophic depopulation and increasing extinction risk in a variety of taxonomic groups (Jenkins et al. 1989; van Riper et al. 2002; Ryan et al. 2008; Blehert et al. 2009). Moreover, epidemics of a novel EID can threaten viability of "common" wildlife species with large geographic distributions (Robinson et al. 2010; Hall and Saito 2008). For example, salmonellosis and avian pox are also recognized as EIDs of wild birds, because their prevalence appears to have increased recently (Tizard 2004; Lawson et al. 2012), particularly in association with bird feeding (Lawson et al. 2010), even if *Salmonella* and avipoxviruses (APVs) seem to be grouped in classical or ordinary pathogens. Both pathogens are considered to have played a role in the decline of wild bird population including common bird species (Tizard 2004; van Riper and Forrester 2007).

Furthermore, *Salmonella* is also known to cause disease in humans, domestic animals and wildlife; however, its significance in public health risks associated with avian salmonellosis as well as in causing wild bird mortality, is not well

understood (Hall and Saito 2008). An outbreak of salmonellosis in humans transmitted from domestic cats (*Felis catus*) associated with predation of *Salmonella* infected wild birds was reported (Tauni and Österlund 2000).

Recently, 75% of human EID pathogens are known to be zoonotic (Taylor et al. 2001). For example, *Encephalitozoon cuniculi* is an obligate intracellular microsporidian that is the causal agent of encephalitozoonosis, increasing attention as an important EID in both humans and animals (Wasson and Peper 2000; Mathis et al. 2005). *E. cuniculi* spores were detected in the urine of one seropositive animal caretaker who was immunocompetent and worked with infected domestic rabbits (*Oryctolagus cuniculus*) (Ozkan et al. 2011). Recently, wildlife is considered as significant reservoirs of *E. cuniculi* infection for both humans and domestic animals (Meredith et al. 2015). Sak et al. (2011) recorded a high prevalence of *E. cuniculi* among both animal keepers and apes in a wildlife center, Cameroon, implying the potential human to ape or *vice versa* transmission.

Consequently, the recent unprecedented increase in the identification of wildlife EIDs (Daszak et al. 2001) has raised concerns that emerging pathogens may represent a substantial threat to global biodiversity as well as domestic animal and human health (Daszak et al. 2000; Smith et al. 2009). Thus, for the prevention and control of emerging pathogens that threaten the One Health, it is important to develop diagnostic methods for wildlife EIDs, to collect the clinical and pathological information, and to understand their epidemiology.

Zoos, which have a wide variety of captive wildlife collections, have been making a great effort to play a powerful role in wildlife conservation to date through *ex situ* breeding programs for endangered species (WAZA 2005). Recently, zoos have identified that one of the 10 most important emerging issues for species conservation is "diseases, zoonoses and biosecurity issues" (Gusset et al. 2014). For good health management of captive animals and public health safety, zoo veterinarians practice routine year-round biosecurity programs such as necropsies on every animal that dies (Janssen and Rideout 2010). Zoo biosecurity should include not only preventive medicine protocols practiced routinely, but also prevention from invasion of pathogens outside into zoos (Travis and Barbiers 2010), because no complete border exists between natural environment and on a zoo ground. Investigation of moribund wildlife and necropsies of the carcasses found on zoo grounds must be useful monitoring tools of wildlife diseases including zoonoses, and provide a baseline measure of the risk posed by local wildlife (Travis and Barbiers 2010). Adler et al. (2011) reviewed about 25 novel records of arthropod-borne EIDs in zoo animals, including a West Nile virus (WNV) infection in a polar bear (*Ursus maritimus*) housed at a Canadian zoo (Dutton et al. 2009), indicating that zoos can function as an early-warning or prediction system for what could happen beyond the zoo environment.

In Japan, there are some case reports of infectious diseases in wild animals found dead on a zoo ground (Asaoka et al. 2004; Nagao et al. 2012) and via

transmission from wild animals to zoo animals (Kondo et al. 1996; Nagao et al. 2012). Currently, a few research institutes conducted surveillance of wildlife infectious agents with collaboration with zoos (Aruji et al. 2004; Ejiri et al. 2009). However, information on wildlife EIDs emerged in Japanese zoos is limited, despite the fact that Japan is one of the countries that have the largest number of zoos. Moreover, few Japanese zoos have conducted investigation of ill or dead wild animals found on zoo grounds as a part of routine biosecurity practices until now.

In the present study, the author has experienced three cases of wildlife EID occurrence, which can affect captive animals, free-ranging wildlife and/or humans, within a zoo facility and in the adjacent natural environment. Thus, these EIDs were investigated using a combination of several diagnostic methods; clinical, pathological, epidemiological and other specific examinations. Furthermore, roles of zoo veterinarians were discussed in advanced health management of valuable zoo animal collections, in public health, and in wildlife conservation. That is, an attempt was made to develop a methodology for zoo biosecurity practices to prevent and control these wildlife EIDs. In chapter 1, a debilitated wild sparrow found on a zoo ground followed by subsequent mass mortality of wild sparrows outside the zoo was investigated to reveal the cause of the death. After the identification of the first sparrow salmonellosis case and *S.* Typhimurium-positive sparrows within the zoo, screening examination of *Salmonella* in zoo-kept animals was conducted as a biosecurity measure. In addition, the mass mortality of sparrows observed in an

outdoor field of the zoo was investigated to evaluate the impact on the local sparrow population by epidemiological field surveys. In chapter 2, an outbreak of emerging avian pox in wild crows including a clinical case of zoo-captive crow with nodular skin lesions was investigated clinically, pathologically, epidemiologically, and by the molecular characterization of 4b core protein (P4b) gene of APVs. The impact on the local crow populations was also evaluated by epidemiological field surveys. In addition, zoo biosecurity practices were conducted to prevent the disease spread to zoo and wild animals. In chapter 3, an outbreak of encephalitozoonosis in a zoo rabbit colony was investigated clinically, pathologically, and epidemiologically. Furthermore, biosecurity measures were conducted to eradicate this zoonotic emerging disease by application of all-out and all-in system for new healthy rabbit colony establishment based on serological survey, and to monitor spill-over to other zoo animals.

CHAPTER 1

Mass mortality of Eurasian Tree Sparrows (*Passer montanus*) from *Salmonella* Typhimurium DT40, winter 2008–09

Introduction

Epizootics of salmonellosis in passerines caused by *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*S.* Typhimurium) (Le Minor and Popoff 1987) have been documented since at least the mid-20th century, with an early report in Great Britain, 1956–66 (Wilson and Macdonald 1967). Recently, salmonellosis has caused widespread mortality in passerines, in countries including North America (Daoust et al. 2000; Hall and Saito 2008), Sweden (Tauni and Österlund 2000), New Zealand (Alley et al. 2002), Norway (Refsum et al. 2003), Great Britain (Pennycott et al. 2006), and Japan (Une et al. 2008).

Salmonellosis is considered an EID of wild birds because its prevalence appears to have increased over the past 40 years (Tizard 2004), particularly in association with bird feeding (Lawson et al. 2010).

In Great Britain, *S.* Typhimurium, with the definitive phage types (DT) 40 and DT56 variant, has been most frequently isolated from wild birds including House Sparrows (*Passer domesticus*) since the 1990s; it was hypothesized that these currently host-adapted strains are circulating and maintained within garden bird

populations (Lawson et al. 2011). Although these 'wild bird' strains have been isolated from various domesticated animals (Alley et al. 2002; Rabsch et al. 2002), they made up less than 0.5% of *Salmonella* isolates detected through livestock surveillance (Pennycott et al. 2006).

Wild birds may transmit *S.* Typhimurium to humans (Hudson et al. 2000; Alley et al. 2002). During epizootics of wild bird salmonellosis, human cases due to DT40 occurred in Scandinavia; wild birds were considered the source of infection, with predation by domestic cats (*Felis catus*) forming the link (Tauni and Österlund 2000).

In winter 2005–06, a mass mortality incident involving 1,517 Eurasian Tree Sparrows (*Passer montanus*) was reported in Hokkaido, Japan. Although several organizations investigated these deaths and inferred the mortality was due to chemical deicer poisoning (Tanaka et al. 2008), salmonellosis (Une et al. 2008), atoxoplasmosis (unpublished data of the author), or *Staphylococcus aureus* infection (unpublished data of Asakawa M), the local government concluded that the cause was unclear.

In winter 2008–09, another mass mortality of tree sparrows occurred in Hokkaido, including an initial case within a zoo. For assessment of the cause of mortality, a comprehensive investigation was conducted. A *Salmonella* screening survey in zoo animals was also conducted for animal hygiene and public health because the association of *S.* Typhimurium infection in zoo birds with House Sparrow

salmonellosis was also reported (Alley et al. 2002; Rabsch et al. 2002).

Here, epidemiological data and field survey results were reported, the influence of this mortality was evaluated on the Eurasian Tree Sparrow populations, and zoo biosecurity measures were discussed.

Materials and Methods

Mortality reports, study areas and specimens

In winter 2008–09, multiple Eurasian Tree Sparrow deaths occurred around Asahikawa, Hokkaido (43°03′51″N, 141°20′49″E), the northernmost island of Japan, where the winter is long and extremely cold, with heavy snowfall and average monthly temperatures below freezing from December through March.

Initially, a sick sparrow found within Asahikawa municipal Asahiyama Zoological Park, was provided to the zoo animal hospital on 11 January 2009 and immediately died. Postmortem examination was performed on the sparrow for a preventive medicine program.

Finally, 202 deaths in 100 incidents at 94 sites were reported by private citizens to Kamikawa Subprefectural Office of Hokkaido Government between December 2008 and April 2009. Twenty-six carcasses were collected mostly by residents, through the government, at 13 sites (12 in Asahikawa, including the zoo, and one in a suburb) between January and April 2009 and were submitted to the zoo for postmortem examinations (Table 1-1).

Epidemiological field surveys

Epidemiological field surveys were conducted at the 13 sites, sparrow information was collected from residents. Wild birds visiting gardens and feeding sites (11 sites including the zoo aviary of Japanese Cranes [*Grus japonensis*] and Oriental Turtle Doves [*Streptopelia orientalis*] where wild Eurasian Tree Sparrows frequently visited to forage) were observed directly for assessment of the sparrow mortality incidents. Questionnaires were set to collect information on dates and numbers of carcasses found, duration of incidents, location, presence of feeding sites, and feeding routines, and earlier episodes of sick or dead birds.

Of the 11 feeding sites, samples of food provided at six sites and composite sparrow feces from seven sites and the zoo, were collected in sterile containers for bacteriological tests. Each sample was thoroughly mixed with a sterile culture swab, Transwab® gel medium with charcoal (Iwaki & Co., Ltd., Tokyo, Japan), and immediately transported to the laboratory.

Postmortem examinations

Postmortem examinations included gross necropsy and virological, bacteriological, toxicological, and histopathological examinations. Tracheal and cloacal swabs were obtained for examinations of avian influenza virus (AIV) (Sakai-Tagawa et al. 2010) and WNV (Stone et al. 2004) using commercial rapid diagnostic test kits (ESPLINE®Influenza A & B-N Fujirebio, Inc., Tokyo, Japan, and VecTest®, Medical

Analysis Systems, Camarillo, California, USA, respectively), following the manufacturers' instructions.

At necropsy, the appearance, age class, body condition, body weight, and pathological findings were recorded. Birds were differentiated as young or adult according to bill color and skull ossification (Svensson 1992). Qualitative body condition scores (emaciated, normal, or fat) were assigned based on visual inspection of pectoral muscle mass and fat deposits between the clavicles. The body weights were compared to those of 11 healthy wild Eurasian Tree Sparrows captured from January to March 2010 with the permission from the Hokkaido Government. Systematic external and internal examinations of body systems were performed and any gross lesions were recorded.

Swab samples from crop, liver, spleen, and cloaca were tested for general bacteria and *Salmonella*. Brain, liver, and pectoral muscle samples were collected and stored frozen for chemical element measurement. Heart, lungs, liver, spleen, kidneys, crop, proventriculus, gizzard, intestines, and brain were routinely examined histopathologically. Sections with gross lesions were Gram-stained. Bacteria from visceral organs were tested in four cases by immunohistochemistry using primary rabbit polyclonal antibody to *Salmonella* O4 antiserum (Denka Seiken Co., Ltd., Tokyo, Japan). Secondary antibody reaction was performed using a peroxidase-conjugated Histofine® Simple Stain MAX PO kit (Nichirei, Tokyo, Japan). Reaction products were visualized using 3'3-diaminobenzidine. The slides

were counterstained with Carazzi's hematoxylin.

Bacteriological examinations

All collected swab specimens were plated onto a selective medium for isolation of *Salmonella*. *Salmonella Shigella* agar (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) was used for fecal samples, and desoxycholate hydrogen sulfide lactose agar (DHL, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) was used for the other samples; all were incubated under aerobic conditions at 37°C for 18–24 hr. Bacterial isolates were identified using colony morphology and Gram staining. Suspect colonies morphologically similar to *Salmonella* spp. were subcultured for biochemical examination and *Salmonella* isolate subspecies were confirmed according to Holt et al. (1994). Serotyping of *Salmonella* isolates was accomplished with commercial O and H antisera (Denka Seiken) in a slide agglutination test according to the method of Popoff and Le Minor (2001).

For *S.* Typhimurium isolates, antimicrobial susceptibility testing was done according to the standards outlined by Clinical and Laboratory Standards Institute (2005) with the following drugs: ampicillin, amoxicillin, piperacillin, amoxicillin-clavulanate, cephazolin, ceftazidime, ofloxacin, trimethoprim-sulfamethoxazol, gentamicin, fosfomycin, fradiomycin, tetracycline, minocycline, chloramphenicol, and colistin.

Molecular typing using pulsed-field gel electrophoresis (PFGE) according to a

previous method (Ribot et al. 2006) and bacteriophage typing according to the method of the Health Protection Agency, London, UK (Anderson et al. 1977), were also performed as described (Izumiya et al. 2005). In PFGE, deoxyribonucleic acid (DNA) of the isolates was digested using infrequently cutting restriction enzyme *Bln*I and *Xba*I (Roche, Mannheim, Germany). The digested DNA of *S. enterica* subsp. *enterica* serovar Braenderup H9812 was used as a molecular marker.

An isolate of *S.* Typhimurium DT40 from a Eurasian Tree Sparrow that died in Sapporo in April 2006 during the last mortality period was used to compare the results of biochemical profiles, PFGE pattern, and phage type.

Determination of chemical elements in tissues

Sodium, calcium, and magnesium, contained in chemical deicers commonly used on the roads in Hokkaido, were determined in the collected tissues using atomic absorption spectrophotometry (AAnalyst 800, Perkin-Elmer, Wellesley, Massachusetts, USA) as described previously (Teraoka et al. 2007). Briefly, approximately 200 mg dried tissue samples were digested in 5 mL nitric acid and 0.5 mL H_2O_2 by heating at 150–200°C in an electronic heater. Digested samples were supplemented with ultrapure water to 5 mL. Nine sparrows that died naturally, collected as live injured cases in 1987–2005 and stored frozen, were used as a normal group.

Salmonella screening of zoo animals as a biosecurity measure

The Asahiyama zoo kept approximately 150 species of animals (750 individuals) including 50 mammal, 90 bird and 10 reptile species. Sixty-five fecal samples were collected from 25 species in 10 facilities frequented by sparrows, or from species that might have had direct contact with affected birds, and subjected to *Salmonella* screening as described above. They included the Japanese Cranes, Oriental Turtle Doves, domestic fowls (*Gallus gallus domesticus*), Pekin ducks (*Anas platyrhynchos domestica*), domestic rabbits, guinea pigs (*Cavia porcellus*), domestic dogs (*Canis lupus familiaris*), timber wolves (*C. lupus* ssp.), Hokkaido brown bears (*U. arctos yesoensis*), polar bears, lions (*Panthera leo*), Amur tigers (*P. tigris altaica*), Amur leopards (*P. pardus orientalis*), a black leopard (*P. pardus* var.), snow leopards (*P. uncia*), spotted seals (*Phoca largha*), Japanese macaques (*Macaca fuscata*), chimpanzees (*Pan troglodytes*), an Ostrich (*Struthio camelus*), Emus (*Dromaius novaehollandiae*), Southern Rockhopper Penguins (*Eudyptes chrysocome*), King Penguins (*Aptenodytes patagonicus*), Nothern Gentoo Penguins (*Pygoscelis papua*), and Humboldt Penguins (*Spheniscus humboldti*). A fecal sample from a wild black rat (*Rattus rattus*) trapped within the crane aviary frequently visited by *S.* Typhimurium-positive sparrows, was also examined.

Population monitoring of Eurasian Tree Sparrows

A population monitoring survey by fixed-radius point count (Ralph et al. 1995)

was conducted as described by Kurosawa et al. (2007) at two sites and compared to the preseason (2007–08) and postseason (2009–10) numbers to evaluate changes in population density. In the population count, the number of sparrows was recorded by the same observer at the same point with a radius of 50 m for 5 min between 8:00 and 10:00 AM. Site A was near Nishikagura Town, where multiple sparrow deaths occurred in 2005–06 and since then the sparrow population had been monitored. Site B was the crane aviary within the Asahiyama zoo, where was chosen to monitor wild sparrows that frequently visited to forage.

Statistical analysis

Differences of body weight and chemical elements in tissues between salmonellosis and normal sparrows were compared by *t*-test with unequal variances using software (JMP version 8.02, SAS Institute, Cary, North Carolina, USA).

Results

Epidemiology of salmonellosis in the sparrow mass mortality

February was the peak month for the number of birds found dead (Fig. 1-1). Using general information on the 26 carcasses as well as gross/histopathological findings and results of virological and bacteriological tests (Table 1-1), all examined dead sparrows were diagnosed as salmonellosis.

Eleven of 13 sites were related to wild bird feeding such as bird tables or sites with food scattered on the ground. Approximately 10 to 100 sparrows visited each feeding site. Other species of bird, including Brown-eared Bulbul (*Hypsipetes amaurotis*), Japanese Tit (*Parus minor*), and Hawfinch (*Coccothraustes coccothraustes*) were also observed; however, their deaths were never reported. All food obtained at six sites was *S.* Typhimurium-negative. At six of eight sites, including the zoo, the fecal samples were *S.* Typhimurium-positive.

Clinical findings of affected sparrows

The initial case within the zoo showed weakness, lethargy, inability to fly, and hypothermia, and died 2 hr after the sparrow was found. Affected birds were observed occasionally at feeding stations by residents. During the survey, four sparrows showing likely related symptoms were observed at two sites. These birds showed lethargy with eyes closed, feathers fluffed up, reluctance to fly, isolation from their flock, and hopping around apparently unaware of their environment; two also showed diarrhea and tenesmus.

Biological information and pathological findings in sparrow carcasses

At necropsy, carcasses showed various degrees of tissue damage from autolysis (Table 1-1). In the intestines of 19 cases (73%), carcass decomposition prevented histopathological examination. Twenty-one sparrows could be differentiated into

relative age classes by their appearance: young (62%) and adult (38%). Of these, between January and February, 76% (13/17) were young. Body condition scores of 92% (24/26) of sparrows that died of salmonellosis reflected emaciation. In contrast, the scores of 11 wild-caught sparrows in the same season were normal or fat. There was a significant difference in body weight between salmonellosis cases (19.4±1.7 g) and healthy sparrows (23.6±1.1 g; $P<0.01$). Of the salmonellosis sparrows, dirty feathers around the cloaca were observed in 56% (14/25).

Characteristic gross findings of salmonellosis cases were pale lesions suggestive of necrosis in the liver (n=18), hepatomegaly (10), congestion in the spleen (11), splenomegaly (7), ingluvitis (14), enteritis (19), and hemorrhage within skull air spaces (13). In 82% (18/22) of cases available for evaluation, scattered necrotic foci were observed on the liver surface; 61% of cases (14/23) had gross lesions consisting of cream-colored plaques of 1–10 mm diameter on the crop mucosa; 86% of cases (19/22), had watery or blood-stained contents (14/19) in the small intestine. A small amount of grain derived from feeding stations was often present in the crop (11/26) and gizzard (14/22).

In the distribution of histopathological lesions (Table 1-1), the majority of birds showed multifocal inflammatory necrosis in the liver (13/13) and spleen (10/16) infiltrated by macrophages, lymphocytes, and plasma cells. Lesions in the crop (7/15) consisted of caseous granulomas or areas of mucosal ulceration containing bacteria colonies surrounded by zones of macrophages and mixed inflammatory

cells. Microcolonies of Gram-negative bacilli were observed in the visceral organs of 11/22 cases and were positive in four cases subjected to immunohistochemistry using *Salmonella* O4 antiserum (crop of Case 2, liver of Cases 4–6, and spleen of Case 5). Lymphocytic infiltration was seen in the kidneys (Cases 11 and 18) and cerebral parenchyma (Case 12). All tested birds (Cases 7–26) were negative for AIV and WNV.

Bacteriological findings of Salmonella

Salmonella Typhimurium was isolated from all examined organs in all necropsied sparrows. All isolates showed a very weak reaction in catalase test and were negative for citrate utilization. Other biochemical characteristics were the same. Twenty-four of 26 isolates (92%) were sensitive to all antimicrobial agents tested. Two isolates showed resistance to ampicillin and amoxicillin and intermediate resistance to piperacillin (Cases 25 and 26).

All isolates had the same PFGE pattern digested with both *Bln*I and *Xba*I except for one smearing with each enzyme (Fig. 1-2). All isolates had the same biochemical profiles and PFGE pattern as the isolate from Sapporo 2006. The *S.* Typhimurium isolates belonged to three phage types of DT40 (88%, 22/25), DT110 (8%, 2/25), and DT120 (4%, 1/25), apart from one case with contamination (Table 1-1).

Concentrations of sodium, calcium and magnesium in tissues

No differences were found in concentrations of sodium and magnesium in brain, liver, and muscle between salmonellosis sparrows and sparrows that died naturally (Table 1-2). Calcium concentrations in the brain and liver in salmonellosis cases were significantly lower than those of normal birds ($P<0.05$).

Salmonella in zoo animals

Salmonella was not detected in any of the 65 fecal samples from zoo animals or the black rat trapped in the crane aviary.

Changes in abundance of tree sparrows at two monitoring sites

Dead sparrows with salmonellosis were collected near two monitoring sites A and B: six (Cases 16–21) and two carcasses (Cases 6 and 7), respectively. Few sparrows were seen between April and October (Fig. 1-3). The number was likely to increase with congregation in groups during winter (November to March) and then to decline between March and April. At site A, sparrows disappeared in February 2009 concurrent with the salmonellosis outbreak. The population size recovered the next winter. At site B, the number declined in March 2009; however, sparrows were still observed in April. Although the population was small in the next winter, it recovered in 2010–11 (data not shown).

Discussion

Septicemic salmonellosis was the main cause of mass mortality in Eurasian Tree Sparrows in Japan during winter 2008–09. Bird feeding sites contaminated with *S.* Typhimurium-positive droppings (e.g., on and beneath bird feeding tables and sites where food was scattered) can be important sources for the exposure to *Salmonella*. This supports previous reports that the prevalence of *Salmonella* infection among wild birds at feeding sites is high (Refsum et al. 2003; Lawson et al. 2010). The same strain of *S.* Typhimurium was detected in droppings excreted by a healthy sparrow visiting a bird table during the field survey, indicating the existence of asymptomatic carriers, which are considered a major source of fatal infections (Daoust et al. 2000; Pennycott et al. 2006).

Mortality peaked during February and March. Asahikawa Local Meteorological Observatory reported an average temperature between January and March of −3.9°C in 2009 versus −5.2°C in 1981–2010. Winter 2008–09 was mild in Asahikawa; the melted snow and unfrozen feces at feeding sites during the day were likely higher-risk factors for fecal-oral transmission from healthy carriers or sick birds. Possible explanations for the winter seasonality include inclement cold weather (which leads to immunosuppression and higher risk of *Salmonella* shedding; Lawson, et al. 2010), tree sparrow ecology (including gregarious and flock-feeding behavior) and human factors (e.g., bird feeding in wintertime, resulting in concentrated populations and increased contact rates; Hudson et al.

2000; Tizard, 2004). In addition, immature sparrows in their first winter may be more affected because of immature immunity. When this outbreak started between January and February, most submitted carcasses were young tree sparrows (76%, 13/17). The proportion of individuals with adult features increased after March (55%, 5/9); they might also have been young sparrows in their first winter because the bill color changes before March, making age identification difficult.

The main isolated *S.* Typhimurium phage type was DT40. Biochemical characteristics, antibiotic-resistance profiles, and PFGE patterns were almost the same in all isolates, indicating a close relationship and a single origin. In addition, this salmonellosis-related mortality may be associated with that in Hokkaido in 2005–06 because the isolates had the same profile as that of the isolate from Sapporo in 2006. This clonal causative DT40 strain may be distributed widely among Japanese tree sparrow populations because a salmonellosis outbreak due to this strain occurred in sparrows in mainland Japan in July 2006 (unpublished data of Une Y).

Although various bird species visited the feeding sites, salmonellosis was not confirmed in other species. Infection mortality may be explained by tree sparrow-specific factors such as particular susceptibility to infection and the synanthropic behavior associated with bird feeding, resulting in greater exposure to *Salmonella* (Refsum et al. 2003), as well as narrow DT40 host-range adaptation (Rabsch et al. 2002; Hughes et al. 2008).

Sparrows with salmonellosis were typically emaciated, consistent with previous reports describing infected birds as having poor body condition (Daoust et al. 2000; Refsum et al. 2003; Lawson et al. 2010). Many birds had enteritis, often with hemorrhage. *Salmonella* Typhimurium-positive bloody droppings were observed around normal sparrow feces on the snow during the field survey, suggesting cases with hemorrhagic enteritis. More than half had dirty feathers around the cloaca, suggesting diarrhea or inability to preen properly due to weakness. Of these, 79% (11/14) had findings of gross enteritis. Affected birds were likely to progress to appetite loss, diarrhea, and dehydration, gradually producing emaciation with subacute disease. The findings differed from a previous report describing House Sparrows with *S.* Typhimurium DT160 infection having good body condition, without diarrhea, and only a few cases with gross intestinal abnormalities (Alley et al. 2002). These differences may have resulted from specificity of host species, such as immunological resistance or variation in the causative *S.* Typhimurium strain. In addition, dirty feathers around the cloaca or bloody droppings in foraging areas can be suggestive of *S.* Typhimurium infection and used as field indicators in addition to poor body condition (Lawson et al. 2010).

Suggestive gross findings such as necrotic lesions of liver and crop in the salmonellosis sparrows, suggesting subacute septicemia, match previous reports (Daoust et al. 2000; Alley et al. 2002; Refsum et al. 2003; Une et al. 2008). Characteristic hemorrhagic lesions within the skull in half of the cases may have

been caused by septicemia, leading to blood coagulation impairment (Daoust et al. 2000). Although the brain was commonly affected by DT160 in House Sparrows (Alley et al. 2002), evidence of infection was rarely seen in this study; only one case showed encephalitis histopathologically.

Salmonella Typhimurium infection is attracting attention as the cause of decline in wild bird populations worldwide (Daoust et al. 2000; Pennycott et al. 2006; Hall and Saito 2008; Lawson et al. 2010). The disappearance of sparrows was observed at monitoring site A, concurrent with a salmonellosis outbreak. Although the population simultaneously declined at site B, this was considered their normal pattern of dispersal behavior. This mortality might have a certain impact on population change, but the influence was considered limited because the population recovered the next season. During the last mortality period (2005–06), a sharp decline in population density was documented in Sapporo (Kurosawa et al. 2006), but it later recovered (Kurosawa et al. 2007). In Japan, Mikami (2009) reported that the sparrow population had dropped by at least half since 1990 and by more than 90% since the 1960s. Further investigation is important to evaluate the potential influence of salmonellosis on the sparrow populations.

In the USA, Hall and Saito (2008) reported that the increased urbanization leading to reduced wildlife habitat or the popularity of bird feeding in residential areas might increase contact between birds and thereby increase opportunities for salmonellosis spread. In this study, the sparrow mortality incidents due to

salmonellosis in 2005–06 and 2008–09 in Hokkaido occurred primarily in Asahikawa and/or Sapporo, the two largest cities. Similarly, anthropogenic feeding to wild birds in such urbanized cities may increase congregation among sparrows, leading to increase contact with other wildlife species, domestic animals and humans, and thereby increase risk for the disease spread and spillover.

There is concern that *S.* Typhimurium may be transmissible from wild birds to poultry and livestock (Alley et al. 2002; Rabsch et al. 2002; Pennycott et al. 2006), and to humans (Hudson et al. 2000; Alley et al. 2002). During the last sparrow mortality period in 2005–06, *S.* Typhimurium DT40 was first recorded in Japan in Eurasian Tree Sparrows (Une et al. 2008) and subsequently cows (*Bos primigenius*; Ito et al. 2010), with the same biochemical characteristics and PFGE pattern, suggesting transmission of DT40 from sparrows to cows. During the sparrow mortality period in 2005–06 and 2008–08, *S.* Typhimurium DT40 has not been detected in any other bird species and also in humans. Therefore, this DT40 strain was considered highly host-adapted to tree sparrows in Japan, thus maintaining a reservoir of infection as previously reported (Hughes et al. 2008; Lawson et al. 2011). This may rarely spillover to other species; however, deeper understanding of the epidemiology of sparrow salmonellosis is important for livestock and human health.

Asahikawa, Sapporo, and the other areas where cow salmonellosis occurred due to DT40 match the Hokkaido Central Flyway of migratory birds. This strain might

have been introduced from overseas endemic areas by migratory birds.

To our knowledge, this is the first report of *S.* Typhimurium DT120 in Japan. The DT110 phage type was isolated from several passerine birds in Norway (Refsum et al. 2002). These limited phage types may suggest that they were variants developed from the major strain, DT40, which was an only isolate detected during the mortality in 2005–06, or they were also introduced from endemic areas. A comprehensive survey of these strains among sparrows across Japan should be performed, to further characterize strain diversity and understand the ecology, by using another genotyping technique such as multilocus sequence typing.

The sodium, calcium, and magnesium levels in the salmonellosis sparrows were not higher than in controls, suggesting chemical deicer poisoning was not involved. Tanaka et al. (2008) reported that such poisoning might be the main cause of mortality in 2005–06; however, chemical elements in carcasses were not previously analyzed. Although sparrows prefer angular, yellowish chemical deicer (Bollinger et al. 2005), no evidence of sparrow ingestion of chemical deicer was found. The reason for significantly lower calcium levels in brain and liver in salmonellosis sparrows was unclear. The normal calcium level should be determined in healthy sparrows, because values varied among control samples, as the SD indicated.

Immediately after the initial salmonellosis case was recognized in the zoo, *Salmonella* screening of zoo animals was conducted and all samples were found negative. Epidemiological investigation, including necropsies of wildlife found

dead on zoo grounds, provides a baseline measure of the disease risk posed by local wildlife and should be an important preventive medicine program for biosecurity.

In conclusion, epidemic infection with *S.* Typhimurium DT40 caused mass mortality of Eurasian Tree Sparrows around Asahikawa, Japan, in winter 2008–09 in the association with anthropogenic feeding to birds. For ecological health, further investigation and continued monitoring of salmonellosis using tree sparrows as sentinels is required to evaluate the impact on sparrow populations, biodiversity, livestock hygiene, and public health, and the relationship to human factors such as bird feeding. Public education regarding conservation medicine and hygiene precautions is necessary to prevent infection spread and to control the disease.

Summary

An outbreak of salmonellosis in wild passerines caused mass mortality of Eurasian Tree Sparrows (*Passer montanus*) in Hokkaido, Japan, 2005–06; however, the etiology was poorly understood. In winter 2008–09, sparrow mortality again occurred in Hokkaido, and 202 deaths in 100 incidents at 94 sites were reported. A comprehensive investigation was conducted to evaluate the cause and impact on sparrow populations. A total of 26 carcasses was collected at 13 sites, including a zoological park. In addition, *Salmonella* screening of zoo animals was conducted as a biosecurity measure. *S.* Typhimurium was isolated

from multiple organs in all examined sparrows; they were diagnosed with septicemic salmonellosis. Eleven sites (85%) were related to wild bird feeding and six of eight sparrow fecal samples, including from the zoo, were *S.* Typhimurium-positive. No infection was detected in the zoo animals. Isolates belonged to three phage types of DT40 (88%), DT110 (8%), and DT120 (4%). Pulsed-field gel electrophoresis patterns were the same in all isolates, regardless of phage types. Biochemical characteristics and antibiotic-resistance profiles of DT40 were similar in all isolates, indicating a single origin. The mortality was likely associated with that in 2005–06 because the isolates had the same profiles. Tissue levels of sodium, calcium, magnesium (the main components of chemical deicer suspected to be the major cause of poisoning deaths in 2005–06 mortality) were not higher in the affected sparrows. Thus, it was concluded that an emerging epidemic infection with *S.* Typhimurium DT40 related to bird feeding was the cause of sparrow mortality in 2008–09, and suggested that this causative strain is host-adapted to sparrows in Japan. The mortality might have some impact on the local population, but its influence was limited.

Table 1-1. Summary data of postmortem findings of 26 carcasses of Eurasian Tree Sparrows (*Passer montanus*) diagnosed as salmonellosis caused by *Salmonella* Typhimurium, winter 2008–09.[a]

Case	Date carcass found (2009)	Location[b]	Feeding site	ST in feeding site	Relative age class	Body mass (g)	Emaciation	Dirty feathers around cloaca	Gross/ histopathologic lesions					Phage types
									Liver	Spleen	Crop	Intestines	Skull[c]	
1	Prior to 10 Jan	A	+[d]	+	Y	20.6	+	−	+/ NA	+/ NA	−/ NE	+/ NA	+	DT40
2	Prior to 10 Jan	A	+[d]	+	Y	18.9	+	+	+/ NA	−/ NE	+/ +[e]	+/ NA	+	DT40
3	10 Jan	A	+[d]	+	Y	20.1	+	+	+/ NA	+/ −	+/ −	+/ NA	−	DT40
4	10 Jan	A	+[d]	+	Y	18.6	+	+	+/ +[e]	−/ NE	+/ +	−/ −	+	DT40
5	11 Jan	A	+[d]	+	Y	18.6	+	+	+/ +[e]	+/ +[e]	−/ −	+/ NA	−	DT40
6	11 Jan	B[f]	+[d]	+	Y	21.1	+	+	+/ +[e]	+/ −	−/ NE	+/ −	−	DT40
7	6 Feb	B[f]	+[d]	+	Y	22.3	−	−	+/ +	+/ +	−/ −	+/ NA	−	DT40
8	7 Feb	C	+[d]	−	Y	17.3	+	−	+/ +	−/ +	+/ +	+/ −	+	DT40
9	8 Feb	D	+	+	A	22.0	−	−	+/ +	+/ +	−/ −	+/ NA	+	DT40
10	11 Feb	E	+[d]	+	Y	19.1	+	−	+/ +	+/ +	−/ +	+/ NA	+	DT40
11[g]	16 Feb	F	−	NE	A	21.3	+	−	+/ NA	+/ −	+/ +	+/ NA	−	DT40
12[h]	16 Feb	F	−	NE	Y	16.7	+	+	−/ NA	−/ NA	+/ +	+/ NA	−	DT120
13	Prior to 24 Feb	G	+[d]	+	Y	21.0	+	+	+/ NE	−/ NE	−/ NE	NA/ NE	+	DT40
14	Prior to 24 Feb	G	+	+	U	19.4	+	+	+/ NE	−/ NE	+/ NE	−/ NE	+	DT40
15	24 Feb	G	+	+	A	19.3	+	+	+/ +	−/ −	+/ +	+/ NA	+	DT40
16	23 Feb	H	+[d]	+	Y	18.7	+	+	+/ +	+/ +	+/ −	+/ NE	+	DT40
17	28 Feb	H	+	+	Y	17.7	+	+	−/ NA	−/ +	+/ NE	+/ NA	+	DT110
18[g]	1 Mar	H	+	+	A	16.0	+	−	+/ +	+/ +	−/ −	+/ −	−	DT40
19	1 Mar	H	+	+	A	21.7	+	+	+/ +	−/ +	+/ NE	+/ NA	−	DT40
20	3 Mar	I	+	−	A	18.9	+	+	−/ +	−/ −	+/ NE	+/ NA	−	DT110
21	3 Mar	I	+	−	A	20.1	+	−	+/ +	+/ +	+/ −	+/ NA	+	DT40
22	7 Mar	J	+	NE	A	17.7	+	+	+/ NA	−/ −	−/ −	+/ NA	+	DT40
23	12 Apr	K	+	NE	U	NE	+	−	NA/ NA	NA/ NA	+/ NA	NA/ NA	NA	contaminated
24	10 Apr	L	−	NE	U	NE	+	NA	NA/ NA	NA/ NA	NA/ NA	NA/ NA	NA	DT40
25	28 Apr	M	+	NE	U	NE	+	−	NA/ NA	NA/ NA	NA/ NA	NA/ NA	NA	DT40
26	28 Apr	M	+	NE	U	NE	+	−	NA/ NA	NA/ NA	NA/ NA	NA/ NA	NA	DT40
% positive or mean values		13 sites	88%[i]	75% (6/8)	Y: 62%	19.4g	92%	56%	86%/ 100%	50%/ 63%	61% / 47%	90.5%/ 0%	59%	DT40: 88%

[a]ST = *Salmonella enterica* subspecies *enterica* serovar Typhimurium; Jan = January, Feb = February, Mar = March, Apr = April; Y = young, A = adult, U =uncertain; DT = definitive phage types; NA = not available for diagnosis because of severe tissue damage after death; NE = not examined; (+) = yes (positive), (−) = no (negative).

[b]A = Kaguraoka; B = Higashiasahikawa (1); C = Suehiro; D = Higashiasahikawa (2); E = Toukou; F = Takasucho; G = Pippu; H = Nishikagura (1); I = Nishikagura (2); J = Higashi 7-6; K = Higashiasahikawa (3); L = Nagayama; M = Higashiasahikawa (4). The numbers after the town names refer to different areas within each town.

[c]Hemorrhagic lesion within air spaces of skull was observed in 13 cases.

[d]Samples of food provided at six sites were collected for ST screening tests. All were negative.

[e]Microcolonies of Gram-negative bacilli observed in tissues were found positive in four cases (Cases 2, 4−6) subjected to immunohistochemistry using *Salmonella* O4 antiserum.

[f]Cases 6 and 7 were collected within a zoo facility in Higashiasahikawa where wild sparrows frequently visited to forage.

[g]Lymphocytic infiltration was seen in two cases (Cases 11 and 18) of kidneys.

[g]Cerebral parenchyma was seen in one case (Case 12).

[g]Fed at 85% of different sites (not fed at F or L).

Table 1-2. Concentrations of sodium (Na), calcium (Ca), and magnesium (Mg) in brain, liver, and muscle obtained from salmonellosis Eurasian Tree Sparrows (*Passer montanus*), Hokkaido, 2008–09 and from those that died naturally.

Values are expressed as means±SD (mg/g dry tissue weight). Values in parentheses indicate case numbers.

Organs	Sparrow groups	Na	Ca	Mg
Brain	Normal[a]	8.8±4.0 (8)	1.4±0.9 (8)	0.6±0.1 (8)
	Salmonellosis[b]	7.7±6.5 (8)	0.4±0.2 (8)*	0.8±0.2 (8)
Liver	Normal	6.4±4.2 (9)	1.0±0.8 (9)	0.9±0.4 (9)
	Salmonellosis	5.7±2.6 (12)	0.2±0.1 (12)*	1.1±0.3 (12)
Muscle	Normal	6.5±5.4 (9)	0.7±0.6 (9)	0.8±0.3 (9)
	Salmonellosis	5.2±2.8 (19)	0.6±0.7 (19)	1.1±0.5 (19)

[a]Wild sparrow carcasses collected as live injured cases between 1987 and 2005 were used for analysis as normal group.

[b]Carcasses of tree sparrows with fatal salmonellosis collected during a period of mass mortality in Japan, winter 2008–09, were used for analysis as salmonellosis group.

* Values are significantly different from normal group ($P<0.05$).

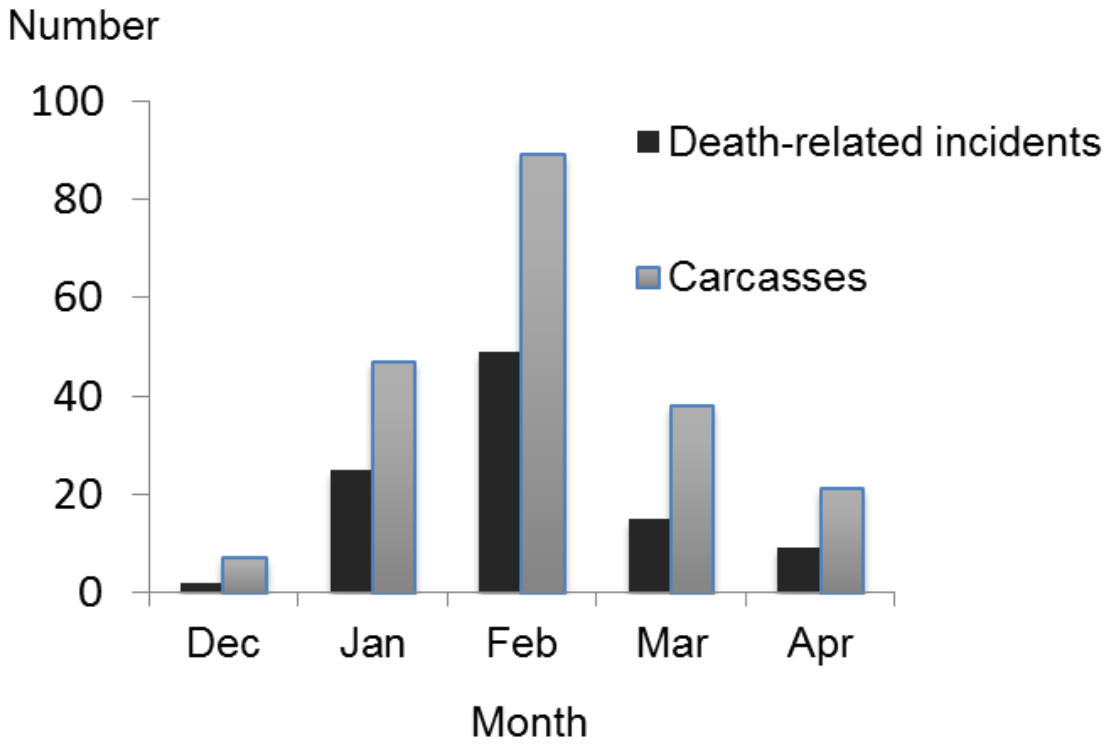


Fig. 1-1. Number of death-related incidents and carcasses of Eurasian Tree Sparrows (*Passer montanus*) by month, reported during a period of mass mortality due to salmonellosis in Hokkaido, winter 2008–09.

Between December 2008 and April 2009, residents reported 202 deaths in 100 incidents at 94 sites.

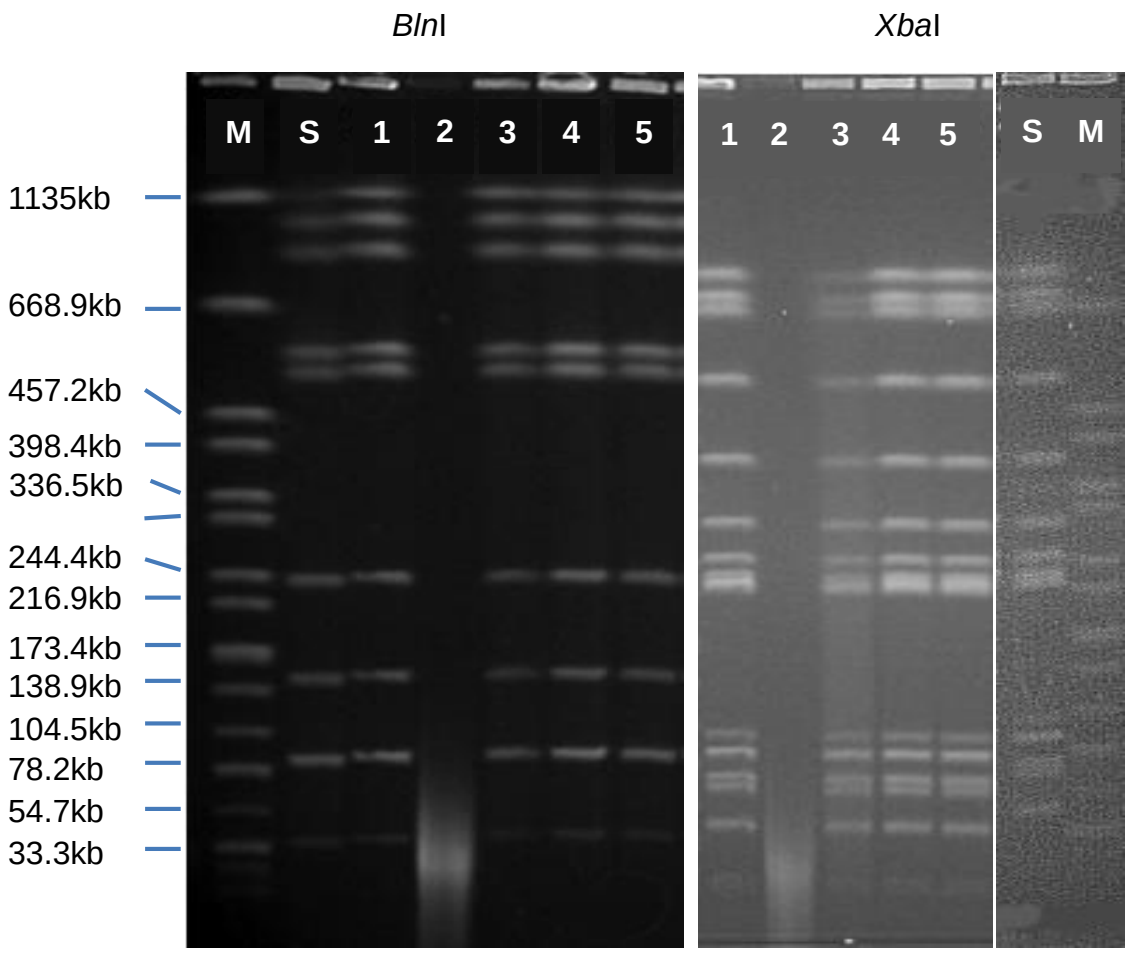


Fig. 1-2. *Bln*I and *Xba*I -digested pulsed-field gel electrophoresis patterns of *Salmonella* Typhimurium isolated from Eurasian Tree Sparrow (*Passer montanus*) samples in Hokkaido, winter 2008–09.

M = a molecular size marker (*S.* Braenderup H9812 digested with *Bln*I and *Xba*I, respectively); S = an isolate of *S.* Typhimurium DT40 from a sparrow that died in Sapporo in April 2006 during the last mortality period; 1–5=Cases 1–5.

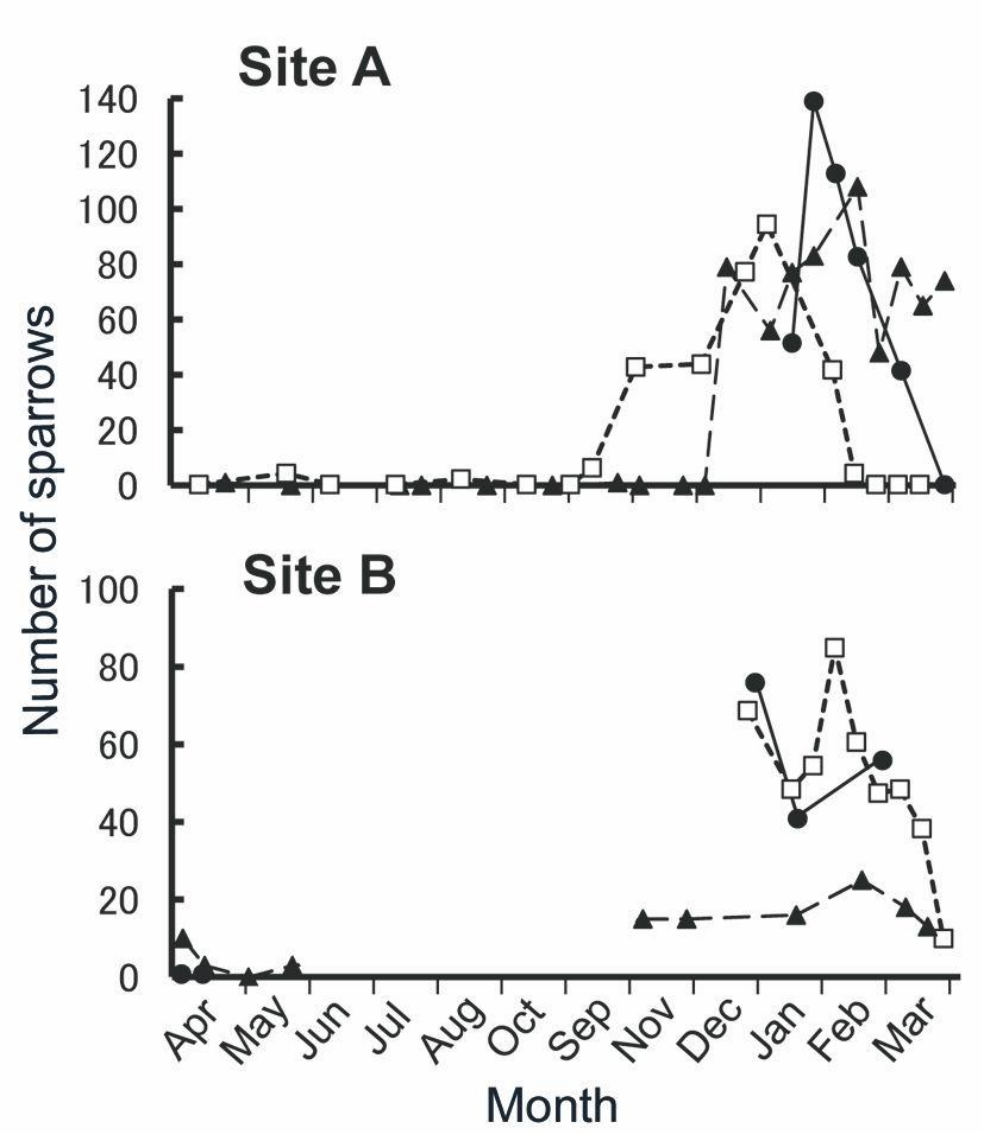


Fig. 1-3. Changes in population abundance of Eurasian Tree Sparrows (*Passer montanus*) counted in 2007–10 at two monitoring sites related to wild bird feeding where carcasses were found during a period of mass mortality due to salmonellosis in Hokkaido, winter 2008–09.

Site A was near the town of Nishikagura. Site B was a crane aviary in the Asahiyama zoo. —●— 2007–08 ···□··· 2008–09 - -▲- - 2009–10.

CHAPTER 2

An epizootic of emerging avian pox in Carrion Crows (*Corvus corone*) and Large-billed Crows (*Corvus macrorhynchos*)

Introduction

Avian pox is an infectious disease caused by double-stranded DNA viruses, belonging to the genus *Avipoxvirus* in the subfamily *Chordopoxvirinae* of the *Poxviridae* family (Skinner et al. 2012). Recently, avipoxviruses (APVs) have been isolated from a wide variety of bird species worldwide (278 host species to date; van Riper and Forrester 2007). There is the possibility of interspecies transmission, as evidenced by phylogenetic analysis based on the APV-specific 4b core protein (P4b) gene (Weli and Tryland 2011; Gyuranecz et al. 2013). The named number of APV species is much smaller than the number of infected bird species, suggesting that further molecular characterization is needed. In Japan, there are a few case reports of avian pox in wild birds (Saito et al. 2009; Watanabe et al. 2009) and captive birds (Yu et al. 2007; Terasaki et al. 2010), but the epidemiology among the Japanese avifauna is poorlystudied.

In most birds, APV infections have been reported as mild, self-limiting and rarelyfatal; however, when lesions occur on eyelids, with secondary infection, or in diphtheritic form, they can be fatal (Tripathy et al. 2000; van Riper and Forrester

2007). Endemic bird populations on islands (e.g., the Hawaiian, Canary and Galapagos Islands) were more negatively impacted by an introduced APV infection than those on continents where the hosts and viruses have had a longer co-evolutionary history (Warner 1968; van Riper et al. 2002; Smits et al. 2005; Parker et al. 2011). Exotic APVs may have played a role in the population decline of Hawaiian Crows (*Corvus hawaiiensis*; Jenkins et al. 1989). In other corvids, there are reports of APV infection (Bolte et al. 1999; Miller et al. 2010; Wheeler et al. 2014), including single case reports pathologically investigated in a Carrion Crow (*C. corone*) (Poulding 1960) and a Large-billed Crow (*C. macrorhynchos*) (Joshi et al. 2012). In Italy, an APV was isolated from a Hooded Crow (*C. corone cornix*), with phylogenetic analysis (Manarolla et al. 2010) based on P4b gene and H3L gene loci (Jarmin et al. 2006). In most parts of Japan, Carrion Crows and Large-billed Crows are common wild birds inhabiting urban and rural areas in the close association with humans, but little is known of avian pox virus infection in these two species.

The present study is the first to report on an outbreak and the subsequent epizootic of avian pox that emerged among the crow populations in Japan in 2006–10 were reported. The clinical and pathological features, the phylogenetic classification of the detected APV P4b genes, and results of assessment of the impact on the local crow populations were documented in this chapter.

Materials and Methods

Mortality reports, study areas and specimens

Between November 2006 and December 2010, more than 29 mortality incidents of crows with proliferative skin lesions were reported by private citizens in Hokkaido. A total of 19 carcasses was obtained to evaluate the cause of death, mainly from Sapporo (Table 2-1). Of these, 13 crows collected between 2006 and 2009 were subjected to a complete necropsy, subsequent histopathological examination and detection of APV gene (Cases 1–13, Table 2-2). The remaining 6 carcasses (Cases 14–19) collected in 2010 were subjected to visual inspection of the lesions.

A total of eight surviving crows with nodular skin lesions were also examined biologically and clinically, and obtained biopsy specimens of the lesions for histopathological examination and APV gene detection. They included a clinical case in aLarge-billed Crow kept for the exhibition at the Asahiyama zoo after being trapped by the Asahikawa City in 2008 (Case 20) and seven crows captured between 2008 and 2009 at banding surveys in Sapporo with the permission from the Hokkaido Prefectutal Government (Cases 21–27).

Biological examinations: Age class, body condition and body mass

Crows were classified into two age groups: juveniles (<1 year old) and adults (>1 year old) (Goodwin 1977; Jenni and Winkler 1994). Body condition was assigned

as emaciated (scored as 1), poor (2), average (3) or good (4) based on visual inspection of pectoral muscle mass and fat deposits between the clavicles. Body masses were compared between avian pox cases and healthy juveniles captured between October and February during 2008–12 (136 Carrion Crowsand 74 Large-billed Crows), and the difference were statistically tested differences Mann-Whitney U test.

Diagnosis of avian pox and evaluation of skin lesions

Avian pox was confirmed histopathologically (van Riper and Forrester 2007) and/or by APV gene detection (Lee and Lee 1997). Additionally, the diagnoses were made based on clinical findings of the characteristic proliferative skin lesions (van Riper and Forrester 2007; Lawson et al. 2012) and epidemiologically.

The number and distribution of skin lesions on various parts of the body were recorded and the severity of infections were scored as mild (1 lesion), moderate (2 lesions) or severe (3 or more lesions, or one lesion on the head) (van Riper et al. 2002).

Pathological examinations

Prior to necropsy, all submitted carcasses were tested for AIV and WNV using the commercial kits, following the manufacturers' instructions. Complete necropsy was performed with subsequent microbiological and histopathological examination.

Skin lesions and tissue samples (skin lesions, heart, trachea, lungs, air sac, liver, spleen, kidneys, proventriculus, gizzard, intestines and brain) and biopsy skin specimens in the surviving crows were examined as mentioned above.

Detection of APV P4b genes and phylogenetic analysis

P4b gene detection was performed by polymerase chain reaction (PCR) on the specimens (Lee and Lee 1997; Saito et al. 2009). DNA from homogenates of the specimens was extracted using SepaGene® (Sanko Junyaku Co., Ltd., Tokyo, Japan). Subsequently, the purified DNA was applied for the PCR with two *Fowlpox virus*-specific primers (P1X [5'-TCAGCAGGTGCTAAACAACA-3'] and P2 [5'-CGGTAGCTTAACGCCGAATA-3']) (P1XP2 PCR). For P4b gene detection of APVs closely related to *Canarypox virus*, another primer pair (P1canF [5'-TGCAAGATTTAATCAGCGTG-3'] and P2canR [5'-GCTTCATCTGATCTCCTATT-C-3']) (canFR PCR) was used. The expected sizes of the amplified DNA fragment were 579 and 183 base pairs long, respectively. The PCR amplifications of the DNA were performed at 94°C for 1 min, 59°C (55°C for canFR PCR) for 1 min and 72°C for 1 min for 35 cycles after initial denaturation for 5 min at 94°C. The PCR products were purified and the DNA sequences were determined using Applied Biosystems® 3130 Genetic Analyzer (Life Technologies Corp., Carlsbad, California, USA). The APV P4b gene sequences detected and obtained from the deoxyribonucleic acid data bank of Japan (DDBJ) were aligned using the

CLUSTAL W in MEGA 6.06 (Tamura et al. 2013). Subsequently, a phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987).

Epidemiological field surveys and population monitoring

To provide an index of avian pox prevalence within the crow populations in Sapporo, fixed spot censuses and banding surveys were conducted.

Fixed spot censuses were conducted between July, when almost all the juveniles had left their nests, and the following February, the last month of the winter roosting season before dispersal for breeding, during 2007–08 and 2011–12 (341 censuses: 10–59 times in each year period) (Reynolds et al. 1980). The censuses focused on spotting diseased individuals and evaluating adults that are rarely trapped at banding surveys. Crows were counted and recorded in terms of their behavior, age and the presence of skin lesions within a radius of 50 m for 5 to 10 min between 8:00 AM and 7:00 PM. The number of crows observed at each census (1–56) was accumulated for calculating proportions of crows with lesions for each year.

Banding surveys were conducted in the suburbs between October and the following February, during which crows assemble in winter roosting sites during 2008–09 and 2011–12 (54 surveys: 9–17 times in each year period). A total of 262 crows (170 Carrion Crows and 92 Large-billed Crows) was captured in traps, and recorded in terms of their age, body mass, general physical condition and the

presence of skin lesions before release. When crows with skin lesions were found, they were sampled. The number of trapped crows at each session (1–17) accumulated for calculating proportions of crows with lesions for each year.

For assessments of the crow populations, the number of incoming crows in the winter roosts was counted from 1–2 hr before sunset until 0.5–1.5 hr after sunset between 2006–07 and 2011–12.

Results

Mortality incidents and distribution

Most of the carcasses (63.2%, 12/19) were found in Sapporo and between September and December (94.7%, 18/19) (Table 2-1). In Sapporo, the first avian pox case (Case 1) was confirmed in 2006. Subsequently, the mortalitie from avian pox increased, peaking in 2007, and then occurring sporadically until 2010. The remaining seven carcasses were collected at two areas in Hokkaido: Hiroo (n=2) in 2008–09 period (Case 10) and in 2009 (Case 12), and Asahikawa (n=5) in 2010. In Hiroo, the 2 carcasses were collected for subsequent examination, although 12 carcasses with the similar skin lesions were found between February 2008 and September 2009.

Biological findings: species, age class, body condition and body mass

All 27 examined crows (13 Carrion Crows and 14 Large-billed Crows) were

juveniles. Of the 13 necropsied carcasses, 76.9% (10/13) were emaciated and in poor body condition (Table 2-2). There were significant differences in body mass between avian pox cases and healthy Carrion Crows (422.7±95.5 g versus 646.1±73.6 g) and Large-billed Crows (527.3±146.3 g versus 773.9±77.9 g) ($P<0.01$) for both comparisons.

Characteristics of skin lesions and clinical findings in affected crows

In the 21 assessed crows (13 carcasses and eight biopsied surviving cases), the severity of infection was scored as 'severe', except for one clinical case assessed as 'moderate' (Table 2-3). In this case (Case 20), the lesions on toes were evaluated as having healed with scars 1.5 month after biopsy.

The majority of lesions occurred on toes (90.5%, 19/21) followed by eyelids (57.1%, 12/21) (Fig. 2-1). Most fatal cases had lesions on the eyelids with three or more digits of the toes (61.5%, 8/13). Only fatal cases had lesions elsewhere (e.g., bill, mouth commissure). In the epidemiological field surveys, two diseased crows (Cases 2 and 6) were found still alive and unable to fly, leading to their capture. They became lethargic and died the next day (Fig. 2-1).

Pathological findings in affected crows, APV P4b genes and diagnoses

Grossly, 26 out of the 27 cases had multiple proliferative wart-like skin lesions. All lesions of the eight biopsied cases were solitary distinct nodules. In contrast,

all lesions of 18 out of the 19 fatal cases were extensively proliferative and aggressive masses, subsequently involving injury, ulceration and necrosis with a putrid odor. The largest coalescent mass lesions measured up to 2.4 cm in diameter (Fig. 2-1).

Histopathologically, 18 out of the 21 crows had epithelial cell hypertrophy and hyperplasia with ballooning degeneration, containing intracytoplasmic Bollinger bodies, and were confirmed as avian pox. In 19 out of the 21 cases, infiltrating bacterial colonies were visible in the ulceration of the keratinized superficial epidermis. In one case (Case 1), hyperplasia and ballooning degeneration of epithelial cells in trachea, bronchi and air sac was observed. All fatal cases had co-morbidities (Table 2-2). Avipoxvirus P4b genes were detected in 15 out of the 21 cases, including three histopathologically avian pox-negative cases. In addition, the remaining six carcasses were assumed as avian pox based on the identical feature of the severe skin lesions themselves. All examined 27 crows were diagnosed as avian pox.

Phylogenetic analysis of detected APV P4b genes

The nucleotide sequences of the APV P4b gene detected from seven specimens (Cases 1, 2, 9, 11, 23, 26 and 20) were submitted to the DDBJ database under the accessions LC055558–64. In six Sapporo specimens (Carrion Crows in 2006 [n=1] and 2009 [n=2]; Large-billed Crows in 2007 [n=1], 2008 [n=1] and 2009 [n=1]), the

nucleotide sequences of the P1XP2 PCR products were identical (Sapporo strain). The P4b nucleotide sequence of the zoo-kept Large-billed Crow specimen (Case 20) differed from those of the Sapporo strain, with 71% similarity (Asahikawa strain). No identical P4b nucleotide sequences of both strains were found in the DDBJ by BLAST search. In the phylogenetic tree (Fig. 2-2), the Sapporo strain was clustered in subclade B1 (Jarmin et al. 2006) with a *Fowlpox virus* isolated from a Hooded Crow (GQ180211) with 99% nucleotide and amino acid similarity. The evolutionary distance was 0.008. In the Asahikawa strain, the closest nucleotide similarity was with an APV isolate from a Macaw (*Ara* spp.; AM050382) in clade C (75%, identity to the other three accessions). The genetic distance was 0.303. The Asahikawa strain formed a single outlier branch with a bootstrap value of 82%.

Avian pox prevalence and number of roosting crows

In the fixed spot censuses, 43.8% (892/2,036) of observed crows were juveniles. All diseased crows were juveniles, except for one adult. Total avian pox prevalence for 5 years was 17.6% (358/2,036) in all crows and 40.0% (357/892) in juveniles. Avian pox prevalence in all crows was highest at 22.7% in 2007–08 and 27.2% in 2008–09 (Fig. 2-3), and decreased to 6.6% in 2011–12. In juveniles, avian pox prevalence was highest at 57.3% in 2007–08 and 52.1% in 2008–09, and decreased to 17.0% in 2011–12.

The majority (93.9%, 246/262) of trapped crows were juveniles (Carrion Crows:

155/170, Large-billed Crows: 91/92), and all diseased crows were juveniles. The total avian pox prevalence for 4 years was 14.5% (38/262): 12.4% (21/170) in Carrion Crows and 18.5% (17/92) in Large-billed Crows. Avian pox prevalence increased from 6.8% in 2008–09 to a peak of 29.7% in 2009–10 (Fig. 2-4), and decreased to 13.1% in 2011–12. No difference in changes of avian pox prevalence was observed between the two species.

The number of roosting crows was stable at around 7,000 to 8,500 between 2002–03 and 2006–07 when the first avian pox case was detected (Fig. 2-5). Then, the crow numbers decreased to 6,133 in 2007–08 and then dropped to 4,680 in 2008–09 as the avian pox prevalence increased, and recovered to around 7,500 in 2009–10.

Discussion

To date, APV infection has been frequently documented in a variety of hosts worldwide (van Riper and Forrester 2007); however, in Japan, APV infection has not been reported in corvids. Herein, an epizootic in 2006–10 of avian pox in crows emerged in Hokkaido, the northernmost island of Japan was documented. To our knowledge, this is the first report of an emerging avian pox outbreak in the Japanese avifauna and in global populations of Carrion Crows and Large-billed Crows.

In Sapporo, since the first identification of an avian pox case in a Carrion Crow

in 2006, mortality incidents by avian pox increased with a peak in 2007. The avian pox prevalence (6.6–27.2%, 17.6% for 5 years) was much higher than those reported for avian pox in wild birds in endemic regions (0.5–1.5%) (van Riper and Forrester 2007). Moreover, the prevalence was similar to those of novel APV introduced in naïve populations on islands (>10%) (van Riper et al. 2002; Smits et al. 2005). In general, avian pox has been reported as mild and rarely resulting in death in most birds (van Riper and Forrester 2007); however, a newly introduced APV can cause severe infection (Warner 1968; Jenkins et al. 1989; van Riper and Forrester 2007). In Hawaiian Crows, it was reported that avian pox could cause direct mortality and skin lesions around the eye and bill, resulting in limited survival (Jenkins et al. 1989; Tripathy et al. 2000). In this study, the APV infection was fatal in juveniles of both species, and most cases were scored as 'severe' infection. In the fatal cases, the skin lesions seemed to have grown distinctively more as large invasive masses than in previous reports (Poulding 1960; Joshi et al. 2012) and the lesions were likely to cause deaths due to disabilities (i.e., impaired vision or inability to feed). Additionally, secondary bacterial infections in the lesions and debilitation caused by the avian pox were considered to lead to immunosuppression and subsequent complications (e.g., fungal infection). Therefore, these results show that a new APV incursion into the endemic avifauna in Sapporo and an epizootic of emerging novel APV infection occurred within the crow population. The reason why almost only juveniles were affected might be

related to their immature immune capacity or the lack of acquired immunity, as previously reported (Buenestado et al. 2004).

The number of roosting crows in Sapporo was approximately 8,000 in 1996 and 1999 (unpublished data of Takenaka M) and it had been stable over the past 10 years; however, an unusual decline in the numbers during 2007–08 and 2008–09 (nearly half of that in 2006–07) was concurrent with the avian pox outbreak. In 2009–10, the number of crows recovered to the previous level, correlated to decreasing avian pox prevalence. In Japan, crow mortality incidents from *Clostridium perfringens*-induced enteritis (Asaoka et al. 2004) and pesticide poisoning (Hosono et al. 2006) were previously reported; however, such unusual crow die-off incidents were not found in Hokkaido during the investigation period. In Hawaii, an exotic emerging avian pox had a negative impact on the population of Hawaiian Crows (Jenkins et al. 1989). Similarly, the outbreak of emerging novel avian pox might have contributed to the unusual decline of the crow population. The number of crows tended to decrease again in 2011–12, but the reason for this decrease is unclear. Further monitoring is important to evaluate the subsequent potential impact on the crow populations because the long-term influence of juvenile mortality on population growth is unpredictable and the APV infection prevalence might be changed by crow influx and dispersal movements.

Commonly, APV transmission can be facilitated mainly by arthropods or direct contact with diseased birds (van Riper and Forrester 2007). In Sapporo, the

mortality incidents from avian pox were concentrated between September and December, correlated with the high prevalence period of avian pox: diseased crows were usually sighted from the end of July to the following March (data not shown). In the Sapporo area, blood-sucking insects (e.g., *Culicidae* mosquitoes, *Culicoides* midges) emerge from May until October with peaks in late July and early August (Kanasugi and Sasaki 1994; Niizuma and Sasaki 1994). Indeed, during the field surveys, these biting insects were observed between June and August, correlating with high avian pox prevalence. Additionally, juvenile crows (including diseased individuals) were observed flocked and foraged together from June with a peak in the population density after they left their natal areas, consistent with a previous report (Tamada and Fujimaki 1993). Family members of the fatal cases tended to have similar skin lesions: in the field observation the collected 13 carcasses, four cases had diseased siblings (n=3) and mother (n=1) of the collected 13 carcasses. Subsequently, the crows assembled at winter roosts from around August, followed by increased avian pox mortalities. Diseased crows were often observed being pecked on the skin lesions by other crows. Therefore, it was possible that the seasonal increase of avian pox was facilitated and enhanced by arthropod vectors (Jenkins et al. 1989; van Riper et al. 2002) and/or by direct contact via sharing the same behavioral patterns (Lachish et al. 2012b), particularly among family members at their nests and congregating sites (e.g., foraging spots or winter roosts). Avian pox in wild birds was reported to have a relatively long initial incubation

period and duration of the disease with up to several months (van Riper and Forrester 2007). In this study, the fatal cases demonstrated chronic clinical and pathological findings. Thus, similarly in avian pox in the crows, it will take some time to develop apparent skin lesions and move into fatal phase. The temporal lag between the peak mortality/ prevalence and the blood-sucking insect emergence or the period of leaving their nests might be explained by a latent period between infection and disease for the APV or the disease progress (Lachish et al. 2012a).

P4b sequence analysis revealed that there are at least two different APV strains affecting crows in Hokkaido. The Sapporo strain was genetically close to a *Fowlpox virus* from a Hooded Crow in Italy (Manarolla et al. 2010). In general, APVs tend to be host family-specific and phylogenetically each clade infects a similarly diverse range of bird hosts (Gyuranecz et al. 2013). Thus, the Sapporo strain may form an APV group that infects corvids as a natural host. The source and route of introduction of the Sapporo strain into the crow population were unclear. However, a new APV could have been introduced via infected migrating birds or vectors (e.g., mosquitoes) (Jenkins et al. 1989; Lawson et al. 2012). The mean annual temperature in Japan has increased by about 1.0°C over the last century, indicating the possibility of a change in the distribution of migrating birds or arthropods (Case and Tidwell 2007). Indeed, recently, new records of wild birds including corvids and an expansion of their range have been reported in Hokkaido (Hokkaido-Block Council of Wild Bird Society of Japan 2007). Thus, it is possible

that the virus was introduced by 1) an overlooked endemic host including corvids in Hokkaido or crows from the main island of Japan, 2) infected birds from overseas migrating into Sapporo, which is located on their East Asian-Australasian Flyway, or 3) infected arthropods.

Interestingly, the Asahikawa strain detected only in a zoo-kept crow seems to be independent from any of the three major clades and considered a novel APV. The severity of infection was scored as 'moderate' only in this case. It is unclear whether the Large-billed Crow is a natural host of this APV, and the source of introduction into the zoo is unknown. In zoos, which house a variety of bird species at much higher densities than in the wild, there is concern that the APV might have spilled over from the other species of captive birds (interspecies transmission) (Weli and Tryland 2011; Gyuranecz et al. 2013). For zoo biosecurity, health management practices was conducted to prevent the disease spread to captive birds in nearby cages as well as to wild birds (e.g., isolation of the patient, screening of captive birds).

In conclusion, an outbreak and epizootic of emerging novel *Avipoxvirus* infection caused juvenile crow mortality and likely led to local population decline in Sapporo, Japan, during 2006–10. Emerging wildlife infectious diseases have been frequently driven by anthropogenic environmental changes (Daszak et al. 2001). In Galapagos finches, human disturbance (i.e., changes in land-use) could have affected immune function in the host species that contribute to APV emergence (Zylberberg et al.

2013). Avipoxviruses can spread more rapidly as host and vector abundance increase (van Riper et al. 2002). In this study, the environmental changes that could have resulted in exposure to stressors were unknown; however, the emerging APV prevalence in the crows could have been enhanced by human factors such as crow congregation in urban areas (e.g., garbage depots) (Becker et al. 2015) or expansion of the range of migrating birds and arthropods influenced by climate change (Fuller et al. 2012). Additionally, the emergence of novel APVs represents a possibility for the incursion of other viruses into Japan, including WNV, which is a significant zoonotic arthropod-borne arbovirus. In the USA, American Crows are routinely utilized as a sentinel for WNV surveillance (Eidson et al. 2005). Similarly, APVs and WNV-sensitive Carrion Crows (Dridi et al. 2013) or Large-billed Crows (Shirafuji et al. 2008) can be useful as an epidemiological model for critical diseases driven by wild birds and arthropods. Therefore, continued monitoring of the APVs in crows and further nationwide APV surveillance based on molecular epidemiology will be important to understand the APV epidemiology in Japanese avifauna, which should contribute to ecological health.

Summary

In 2006–10, an epizootic of emerging avian pox occurred in Carrion Crows (*Corvus corone*) and Large-billed Crows (*C. macrorhynchos*), leading to mortality of juvenile crows in Hokkaido, the northernmost island of Japan. A total of 27 crows

with proliferative skin lesions (19 carcasses and 8 biopsied cases [one in zoo captivity]) was diagnosed as avian pox clinically, histopathologically, by detection of Avipoxvirus (APV)-specific 4b core protein (P4b) gene, and epidemiologically. The fatal cases demonstrated intensively severe infection and aggressive lesions with secondary bacterial infection. Since the first identification of avian pox in Sapporo in 2006, the frequency of mortality events has increased, peaking in 2007–08. Mortalities have subsequently occurred in other areas, suggesting disease expansion. In Sapporo, prevalence of avian pox evaluated by field censuses during 2007–12 was 17.6% (6.6–27.2%), peaked during 2007–08 and 2008–09, and then decreased. All diseased crows were juveniles, except for one adult. The number of crows assembling in the winter roosts had been stable for over 10 years; however, it declined in 2007–08, decreased by about 50% in 2008–09, and recovered to the previous level in 2009–10, correlated with the avian pox outbreak. Thus, avian pox probably contributed to the unusual decline of the crow population. All P4b sequences detected in six specimens in Sapporo were identical and different from any previously reported sequences. Interestingly, the sequence detected in the zoo-kept crow was distinct from any of reported clades, and thus interspecies transmission was suspected. This is the first report of an emerging novel avian pox outbreak in the Japanese avifauna and in global populations of Carrion Crows and Large-billed Crows. Longitudinal monitoring of the prevalence of this emerging APV is needed to evaluate its impact on the crow population.

Table 2-1. Summary of 19 crow carcasses with avian pox-suspected skin lesions.[a]

Examination	Necropsy (Cases 1–13 in 2006–09)	13 (11)
	Visual inspection (Cases 14–19 in 2010)	6 (1)
Species	Carion Crows	10 (5)
	Large-billed Crows	9 (7)
Location[b]	Sapporo	12
	Hiroo	2
	Asahikawa	5

Year	2006–07	1 (1)
	2007–08	6 (6)
	2008–09	3 (2)
	2009–10	3 (2)
	2010–11	6 (1)
Month	September	4 (3)
	October	7 (7)
	November	3 (2)
	December	4 (0)
	April	1 (0)

[a]Values indicate numbers of collected crows. Values in parentheses indicate those of in Sapporo.

[b]Sapporo (43°03''43''N, 141°21'16''E); Hiroo (42°17'10''N, 143°18'42''E, approximately 270km southeast of Sapporo); Asahikawa (43°46'15''N, 142°21'54''E, approximately 130 km northeast of Sapporo).

Table 2-2. Necropsied 13 crow carcasses, diagnosed as avian pox.[a]

Case	Date found carcass		Species[b]	Location[c]	Sex[d]	Body condition[e]	Body mass (g)	Co-morbidities and other histopathologic findings[f]	APV[g]
	Month	Year							
1	Nov	2006	*Cc*	S	M	3	570	SBI, Hyperplasia and ballooning degeneration of epithelial cells in tracheal, bronchial and air sac, Congestion in lung	+
2	Sep	2007	*Cm*	S	F	2	457	SBI, HNE (*Clostridium perfringens* infection), Airsacculitis, Hepatic necrosis	+
3	Oct	2007	*Cc*	S	U	1	317	SBI, Hepatic necrosis	−
4	Oct	2007	*Cc*	S	M	1	325	SBI, Airsacculitis, Hepatic necrosis	+
5	Oct	2007	*Cm*	S	M	1	482	SBI, Airsacculitis, HNE (*C. perfringens* infection suspcted)	+
6	Oct	2007	*Cm*	S	F	2	470	SBI, FI in air sac (*Aspergillus* infection suspected), Hepatic necrosis	+
7	Nov	2007	*Cm*	S	M	1	428	SBI, FI in liver (*Aspergillus* infection suspected)	+
8	Sep	2008	*Cm*	S	M	4	850	Aspiration pneumonia, Systemic congestion, Hepatic necrosis	+
9	Oct	2008	*Cm*	S	F	2	464	SBI, Septicemia, FI in gizzard (*Candida* infection suspected)	+
10	Apr	2009	*Cc*	H	M	1	430	SBI, Airsacculitis, Septicemia	−
11	Sep	2009	*Cc*	S	U	3	478	SBI, Hemorraharge in lung	+
12	Sep	2009	*Cc*	H	M	1	416	SBI, Aspiration pneumonia	−
13	Oct	2009	*Cm*	S	F	2	540	SBI, HNE (*C. perfringens* infection suspcted), Necrotic hepatitis	−

[a]Avipoxvirus (APV) infection was confirmed based on characteristic histopathologic findings (Bollinger body). One case (Case 8) demonstrated inactive avian pox lesions after the masses fell off (skin defects and necrosis in the adjacent bone); however, it was diagnosed as avian pox by detection of APV-specific 4b core protein (P4b) gene.

[b]*Cc* = Carrion Crow (*Corvus corone*); *Cm* = Large-billed Crow (*C. macrorhynchos*).

[c]S = Sapporo; H = Hiroo.

[d]M = male; F = female; U = unknown.

[e](1) = emaciated; (2) = poor; (3) = average; (4) = good.

[f]SBI = secondary bacterial infection in the lesions; HNE = hemorrhagic necrotic enteritis; FI = fungal infection.

[g](+) = APV P4b gene positive; (−) = APV P4b gene negative.

Table 2-3. Skin lesions in 21 crows, diagnosed as avian pox.

		Carcasses (n=13)[a]	Biopsied cases (n=8)[b]	Total (n=21)
Severity of infections	Moderate	0	1	1
	Severe	13	7	20
Distribution	Toe	12	7	19
	Eyelid	9	3	12
	Bill, cere and mouth commissure	5	0	5
	Foot	4	0	4
	Bend of wing	1	0	1
	Other head region[c]	4	0	4
Gross findings	Proliferative skin lesion[d]	12 (masses)	8 (nodules)	20
Histopathologic findings	Bollinger body[e]	12	6	18
APV P4b gene	P1XP2 PCR; canFR PCR	7; 9	3; 6	10; 15

[a]Cases 1－13.

[b]Cases 20－27.

[c]Other head region included jaw, crown and neck.

[d]One case demonstrated inactive lesions (Case 8).

[e]Three cases demonstrated no Bollinger bodies. One case (Case 8) was diagnosed having necrosis in the adjacent bone. Two biopsied cases (Cases 24 and 26) involved suppurative dermatocellulitis.

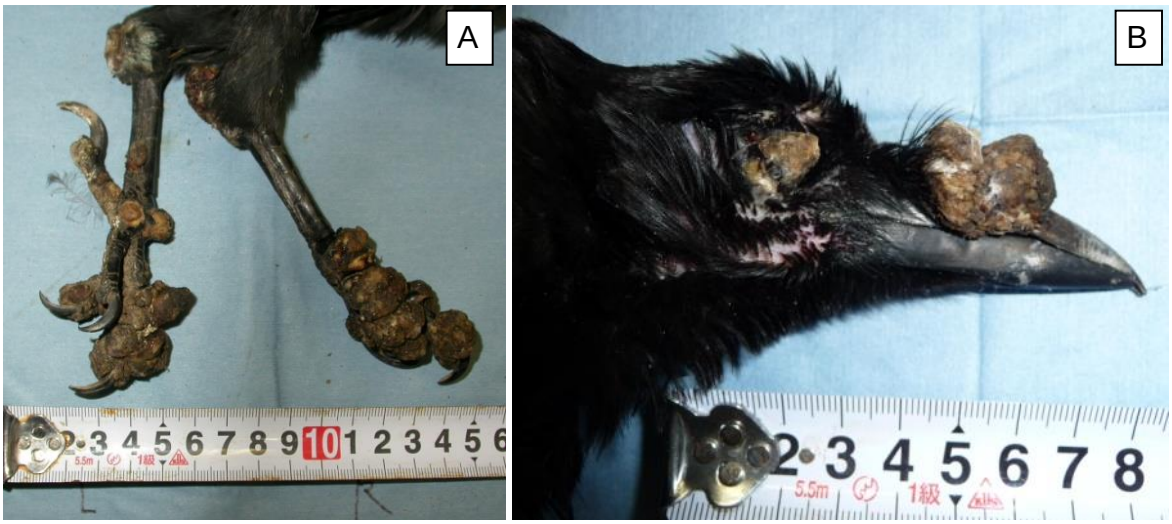


Fig. 2-1. Gross findings of skin lesions in crows with *Avipoxvirus* infection.

Nodular and proliferative coalescent masses grow: (A) on both feet and toes, leading to an inability to walk, in a Large-billed Crow (*Corvus macrorhynchos*) carcass collected in Sapporo, in October 2007 (Case 6); (B) on both right upper and lower eyelids, leading to impaired vision, and cere, infiltrating into the nasal cavity, in a Carrion Crows (*C. corone*) carcass collected in Hiroo, in September 2009 (Case 12).

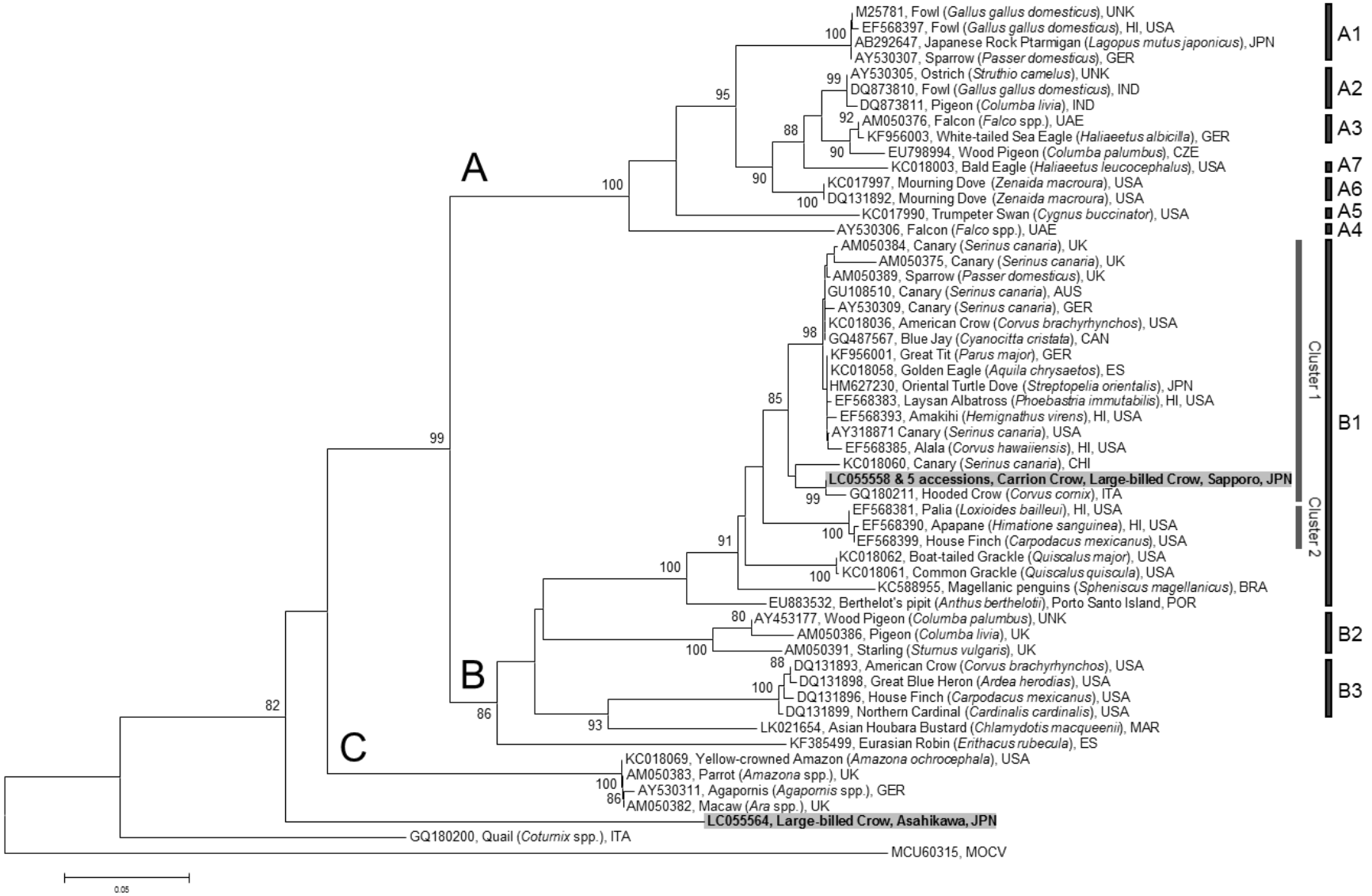
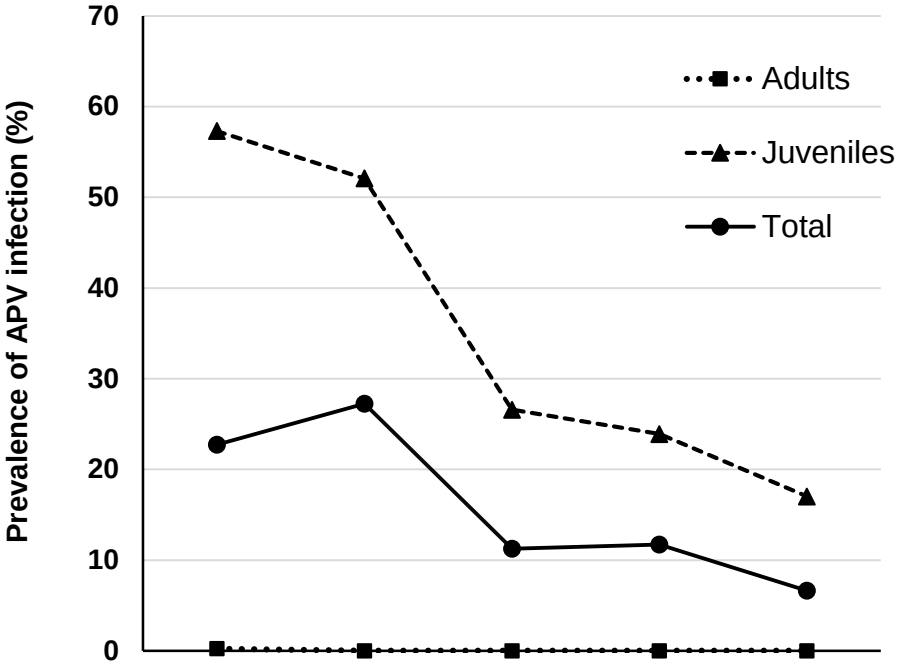


Fig. 2-2. Neighbor-joining phylogenetic tree based on DNA sequences of *Avipoxvirus* (APV)-specific 4b core protein genes detected in crows in Japan and other isolates downloaded from the DDBJ database.

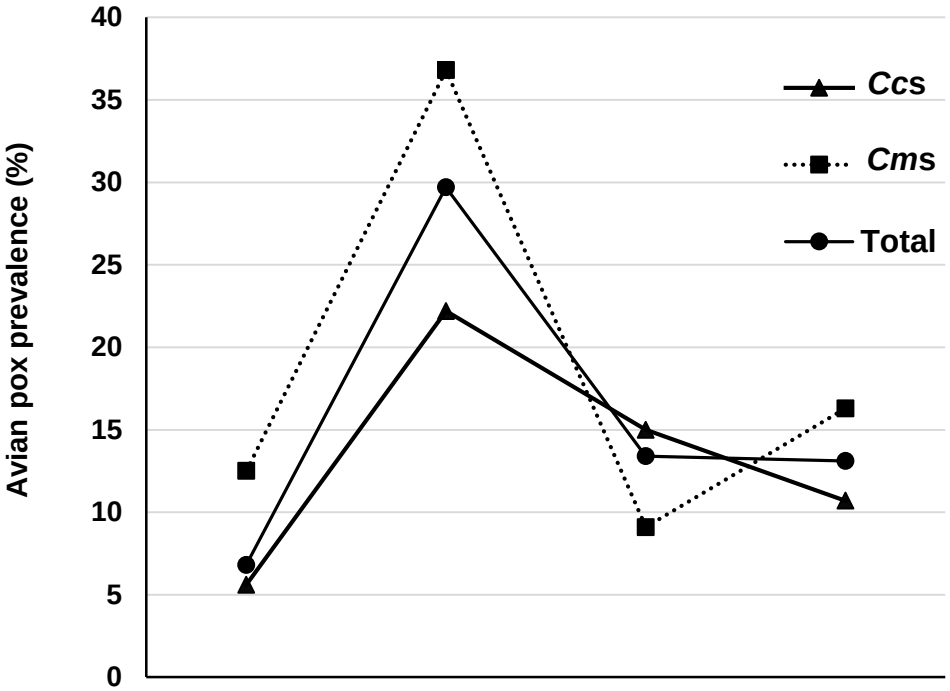
Sequences of the *Molluscum contagiosum virus* (MOCV: MCU60315) P4b gene were used as an outgroup. APV clades A, B and C with subclades A1–7 and B1–3 are labeled according to previous reports (Jarmin et al. 2006; Gyuranecz et al. 2013). Bootstrap values for 1,000 replicates (posterior probability values of >80) are shown at the respective nodes. Bar indicates one substitution per 100 nucleotides. Isolate accession numbers, hosts and origins are indicated with the following abbreviations: Austria (AUT), Brazil (BRA), Canada (CAN), Czech Republic (CZE), Chile (CHI), Germany (GER), Hawaiian Islands (HI), India (IND), Italy (ITA), Japan (JPN), Morocco (MAR), Portugal (POR), Spain (ES), United Arab Emirates (UAE), United Kingdom (UK), United States of America (USA) and unknown (UNK). The APVs detected in Carrion Crows (*Corvus corone*) and Large-billed Crows (*C. macrorhynchos*) in the present study are highlighted in gray.



Year	2007–08	2008–09	2009–10	2010–11	2011–12
Adults	1/421	0/175	0/236	0/165	0/147
Juveniles	157/274	100/192	46/173	38/159	16/94
Total	158/695	100/367	46/409	38/324	16/241

Fig. 2-3. The proportions of crows with avian pox-suspected skin lesions are shown as avian pox prevalence, including Carrion Crows (*Corvus corone*) and Large-billed Crows (*C. macrorhynchos*), counted by fixed spot censuses in Sapporo, between July and February in 2007–12.

Values below year indicate number of diseased individuals/ counted individuals in adult, juvenile and total crows, respectively.



Year	2008–09	2009–10	2010–11	2011–12
Ccs	2/36	4/18	9/60	6/56
Cms	1/8	7/19	2/22	7/43
Total	3/44	11/37	11/82	13/99

Fig. 2-4. The proportions of crows with avian pox-suspected skin lesions are shown as avian pox prevalence of Carrion Crows (*Corvus corone*: *Ccs*) and Large-billed Crows (*C. macrorhynchos*: *Cms*), captured at banding surveys in the suburbs of Sapporo, between October and February, 2008–12.

The majority (93.9%, 246/262) of trapped crows were juveniles. All diseased crows were juveniles. Values below year indicate number of diseased/ banded crows.

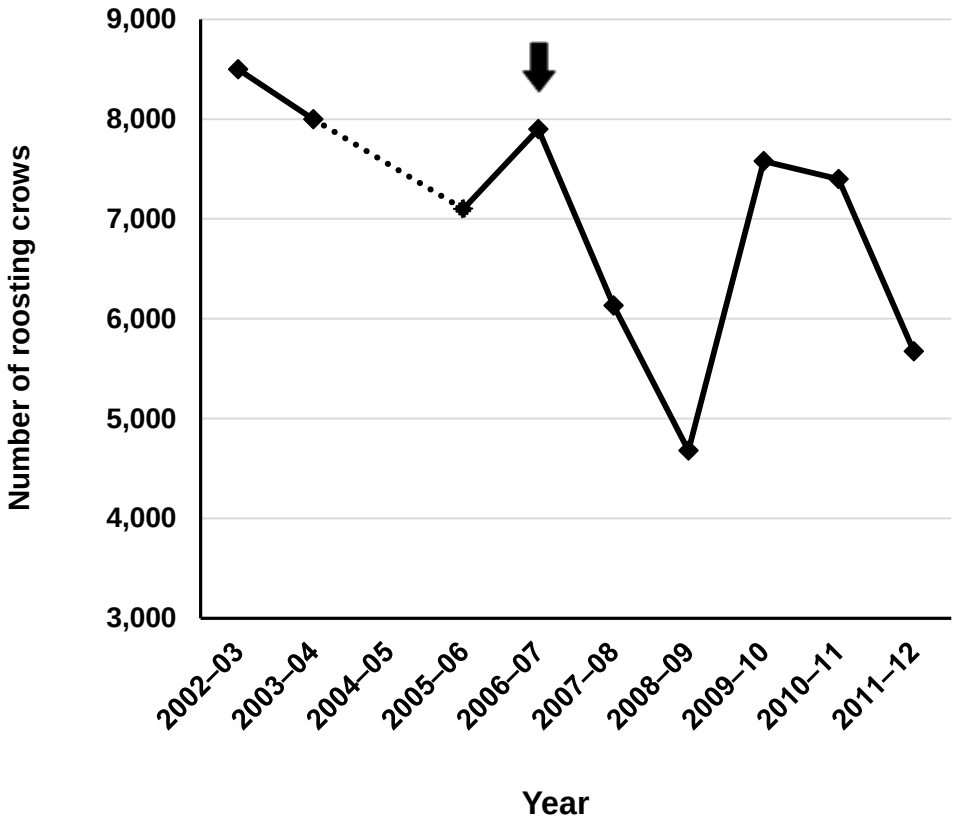


Fig. 2-5. Changes in number of roosting crows, including Carrion Crows (*Corvus corone*) and Large-billed Crows (*C. macrorhynchos*), counted in the winter roosts in Sapporo, between 2006–07 and 2011–12 (on 11 February, except for on 19 January, in 2007–08).

Our previous data between 2002–03 and 2005–06 are also indicated for comparison (not obtained in 2004–05). The period when the first carcass was diagnosed as avian pox is shown by an arrow.

CHAPTER 3

Surveillance for an outbreak of *Encephalitozoon cuniculi* infection in rabbits (*Oryctolagus cuniculus*) housed at a zoo and biosecurity measures

Introduction

Encephalitozoon cuniculi is an obligate intracellular microsporidian parasite (Canning and Lom 1986; Wasson and Peper 2000; Mathis et al. 2005). Spontaneous *E. cuniculi* infections have been documented in various host species including laboratory and domestic rabbits, guinea pigs, goats (*Capra hircus*), sheep (*Ovis aries*), horses (*Equus caballus*), carnivores and primates (Canning and Lom 1986; Boot et al. 1988; Wasson and Peper 2000; Mathis et al. 2005). Guinea pigs housed with rabbits are reported to have a particularly high risk of infection with *E. cuniculi* (Boot et al. 1988). In recent years, severe diseases have become recognized more frequently in pet rabbits (Valencakova et al. 2008; Jeklova et al. 2010; Künzel and Joachim 2010).

Furthermore, *E. cuniculi* has emerged as a significant opportunistic pathogen of immunocompromised humans, particularly acquired immunodeficiency syndrome (AIDS) patients. It has been indicated as a zoonotic pathogen (Didier et al. 1995; Deplazes et al. 1996; Rossi et al. 1998; Mathis et al. 2005), and the number of

records of patients with *E. cuniculi* infection has been increasing worldwide with the increase in human immunodeficiency virus (HIV)-infected patients. *E. cuniculi* is listed in a 1996 WHO report as an emerging infectious agent (World Health Organization 1996). There was also a report documenting that an HIV-uninfected immunocompetent laboratory worker was accidentally infected with *E. cuniculi* when drops containing spores were spilled in his eyes (van Gool et al. 2004). Moreover, *E. cuniculi* spores were detected in the urine of one seropositive animal caretaker who was immunocompetent and worked with infected rabbits (Ozkan et al. 2011). In addition, in Japan, specific immunoglobulin G (IgG) and IgM antibodies against the polar tube of *E. cuniculi* were detected in HIV-infected individuals and even in healthy people (Omura et al. 2007). Nevertheless, only one case of natural *E. cuniculi* infection in a child has been reported in Japan (Matsubayashi et al. 1959). However, this case was considered not to have been unambiguously attributed to *E. cuniculi*, as species differentiation was not possible at that time (Mathis et al. 2005). Thus, the prevalence of *E. cuniculi* in humans is still unclear in Japan.

In Japan, rabbits have become a popular companion animal, and a variety of improved breeds, especially dwarf-breeds, have been imported from the USA and Europe, where high seroprevalence of *E. cuniculi* in rabbits has been shown by serological surveys (Greenstein et al. 1991; Keeble 2001; Hacourt-Brown and Holloway 2003; Valencakova et al. 2008; Jeklova et al. 2010; Künzel and Joachim

2010). Furthermore, in Japan, high seroprevalence of *E. cuniculi* in pet rabbits was reported (Igarashi et al. 2008). *E. cuniculi* infection has also been reported recently in squirrel monkeys (*Saimiri sciureus*) (Asakura et al. 2006) and domestic dogs (Sasaki et al. 2011) living around humans in Japan. This background is associated with the need for an increased focus on public health issues and has prompted us to perform systemic studies on *E. cuniculi* (Furuya 2009). Pathological studies and case reports of encephalitozoonosis in laboratory and pet rabbits have also been increasing in number in Japan (Takano et al. 1993; Park et al. 2007; Igarashi et al. 2008; Furuya 2009). However, there is still little information based on comprehensive evaluation of the disease by systemic epidemiological surveys in conjunction with clinical, histopathological and serological studies in Japan.

Furthermore, efforts to eradicate *E. cuniculi* from a rabbit colony were reported to be successful by using a serological survey in facilities overseas in which outbreaks occurred (Cox et al. 1977; Bywater and Kllett 1978; Boot et al. 2000). However, there have been no reports on hygiene measures to assist in the prevention and control of disease after an outbreak of encephalitozoonosis in Japan.

In this chapter, the results of clinical, pathological and serological surveys of an outbreak of encephalitozoonosis that occurred in a zoo rabbit colony in Japan, where the disease had never been seen for 30 years since the opening of the zoo, were described. In addition, biosecurity measures that successfully prevented the disease spread and eradicated this disease within the zoo were reported.

Materials and Methods

Specimens

In an educational facility at the Asahiyama zoo, 42 rabbits were housed and exhibited for animal contact experiences. The zoo kept approximately 150 species of animal (750 individuals), including 50 mammals, 90 birds and 10 reptiles.

Initially, two juvenile rabbits showed symptoms of central nervous system dysfunction and were sent to the animal hospital of the zoo on 5 and 8 May 1999. They died on 9 and 20 May, respectively. They were pathologically diagnosed as encephalitozoonosis. From the onset until 15 March 2001, all rabbits were examined for an epidemiological surveillance study of *E. cuniculi* infection.

The rabbit breeds were as follows: 19 Lop-eared rabbits (Lops), 7 Angora lops, 5 Lion lops, 5 Lions, 3 Netherland dwarfs, a Flemish giant, a Lion-Angora mixed breed and a mixed breed. Of these, 38 rabbits were subjected to serological examination. A total of 21, including 4 cases that died and 17 cases euthanized due to suspected encephalitozoonosis on the basis of clinical or serological diagnosis, was examined pathologically. All examined animals were handled according to the guidelines for laboratory animal handling published by the Fund for the Replacement of Animals in Medical Experiments (FRAME) (Balls M 1994). Euthanasia was performed humanely by intravenous administration of saturated potassium chloride solution after anesthesia by injection of an excess of ketamine hydrochloride.

Clinical and pathological examinations

The histories of each rabbit were taken from zookeepers, and general physical conditions such as behavior, appetite and body-weight changes were checked. A complete physical examination including inspection, auscultation and palpation was performed. In addition, further diagnostic procedures, such as blood test, urine analysis, radiography of tympanic bullae and abdominal ultrasound examination, were performed to rule out other differential diagnoses, if necessary. Complete necropsy and histopathological examination were performed on all of the 21 individuals mentioned above. Organs including brain, kidney, heart, lung, liver, spleen, duodenum, ileum and colon were examined as described above.

Serological examinations for E. cuniculi infection

In the 38 rabbits, to detect specific antibodies against *E. cuniculi,* enzyme-linked immunosorbent assay (ELISA) capable of measuring IgM and IgG antibodies (n=15) and western blot assay (WB) (n=38) were performed using purified *E. cuniculi* spores as an antigen (Igarashi et al. 2008). Briefly, the spores were propagated *in vitro* using rabbit kidney cell (RK-13) cultures, isolated from culture supernatants and purified by density gradient centrifugation with Percoll (GE Healthcare BioSciences AB, Uppsala, Sweden) (Furuya et al. 2001; 2008). Microtiter plate wells were coated with the extracted soluble antigen at a concentration of 2 μg/ml. To measure anti-*E. cuniculi* IgG antibodies in serum

samples using the IgG-ELISA test, a 100 µl volume of rabbit sera diluted at 1:400 in phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) was added to each well and incubated for 1 hr at room temperature. Bound antibodies were probed with a 100µl volume of horseradish peroxidase (HRP)-conjugated protein A (Kirkegaad & Perry Laboratories, Gaithersburg, Maryland, USA) diluted at 1:4,000 in PBS-T by incubating for 1 hr at room temperature. In the IgM-ELISA test, serum samples were diluted at a 1:50 ratio in PBS-T and HRP-conjugated anti-rabbit IgM (mu-chain specific; Southern Biotechnology Associate, Birmingham, Alabama, USA) was used at a 1:4,000 dilution. The enzyme activity of bound peroxidase was revealed by adding 100 µl of the 2,2'-azino-bis (3-etylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) substrate (Zymed Laboratories Inc., San Diego, California, USA) to each well. Optical density (OD) was measured at 415 nm in each well of microtiter plates using a microplate reader, and the results were regarded as positive when OD values were 1.0 or more according to the extent of absorbance.

WB procedures were performed as described previously (Weiss et al. 1992). Briefly, for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the soluble antigen (approximately 50 µg of protein per lane) was electrophoresed using 10% polyacrylamide mini-gels under reducing conditions (Furuya et al. 1995) and transferred to nitrocellulose membrane. Serum samples were diluted 1: 200 in PBS-T before the assay. Membrane samples were incubated with the primary

antibodies (100 µl volume of serum samples) for three hours then with the secondary antibodies (alkaline phosphatase labeled anti-rabbit IgG diluted 1: 1,000 in buffer) for two hours at room temperature. The substrate used was 5-bromo-4-chloro-3-indolylphosphate (BCIP) / nitrotetrazolium blue (NBT) solution. A positive judgment was evaluated according to the presence of band formation, that is, if there was no band formation, the sample was considered negative. If judgment was difficult, it was interpreted as false positive, and 1+, 2+ and 3+ scores were defined depending on the number of bands. To rule out toxoplasmosis, serum assays for anti-*Toxoplasma gondii* antibodies were carried out on seven individuals using the latex agglutination method (Toxocheck-MT "Eiken", Tanabe, Osaka, Japan).

Biosecurity measures with epidemiological surveillance for control of E. cuniculi

Biosecurity measures were carried out for animal hygiene and public health after the first two cases of encephalitozoonosis from 1999 until 2001. The procedures included monitoring, surveillance, isolation, transport limitation, elimination, eradication, prevention and staff education. Encephalitozoonosis was monitored by clinical, serological and pathological examinations in the rabbit colony. Rabbits with suspected *E. cuniculi* infection were immediately isolated and humanely euthanized for epizootic prevention. Epidemiological surveillance, including analysis of the history of rabbit introduction, was carried out to estimate the source

of the infection. All clinical cases of rabbits in this colony presented to the animal hospital were evaluated retrospectively over two years.

Besides rabbits, three guinea pigs with suspected encephalitozoonosis were pathologically examined during this period; two cases showed fatal weakness, and one euthanized case showed stunted growth and neurological signs like convulsion. A Hokkaido mountain hare (*Lepus timidus ainu*), which was housed separately from the rabbit colony, showed neurological symptoms such as ataxia and hind-limb asthenia (partial paralysis), and was pathologically investigated after its death. One goat, one pig (*Sus scrofa domesticus*), three ferrets (*Mustela furo*) and three hedgehogs (Erinaceidae) that were housed in the same area as the rabbit colony died after various stages of progression and were also pathologically examined.

Results

Clinical findings of affected rabbits

Eleven out of 42 rabbits (26.2%) showed various clinical symptoms attributed to encephalitozoonosis (Table 3-1). They were aged from 40 days to four months (juveniles; n=5) and over four months (adults; n=6). The most affected breed with clinical symptoms was Angora lops (n=5/7). The affected rabbits showed depression (n=9), ataxia (n=7), torticollis (n=5), stunted growth (n=5), death (n=4) and hind-limb asthenia (n=3). There were two major groups that presented characteristic

clinical signs: (a) The first two juvenile cases showed stunted growth, depression, ataxia and hind-limb asthenia, followed by eventual death (Fig. 3-1); (b) five cases showed sudden torticollis and ataxia.

Seroprevalence of E. cuniculi infection in the rabbit colony

Of 15 rabbits examined by ELISA, 93.3% (14/15) and 60.0% (9/15) were specifically IgG and IgM antibody-positive, respectively. Twenty-seven out of 38 rabbits (71.1%) were seropositive by WB: three were 1+, five were 2+ and 19 were 3+. It was found that all of the serum samples showing OD values over 1.0 in the IgG-ELISA test generated distinct multiple bands in WB, whereas one sample showing OD values below 1.0 revealed no bands stainable by WB. All of the seven rabbits (Cases 3–9) tested for anti-*Toxoplasma gondii* antibodies were negative.

Pathological findings

All of the 21 rabbits revealed infection with *E. cuniculi*. The pseudocysts were detected scattered throughout the brain in 16 cases (Table 3-1), in which many gram-positive organism bodies were observed. At necropsy, all cases showed small white spots or focal depressions of several millimeters in diameter on the kidney cortex. Histopathologically, 20 cases had mild to moderate chronic interstitial nephritis and nonpurulent encephalitis with glial nodules or granulomas. The remaining one case had an anemic infarct in the kidney, encephalomalacia and

moderate perivascular mononuclear cell cuffs in the brain. The heart (61.9%), lung (85.7%), liver (81.0%) and spleen (71.4%) were also shown to be involved in proliferating inflammatory changes with mononuclear cell infiltration. In addition, severe coccidia infection was found in the intestines of one case.

Comprehensive evaluation of E. cuniculi infection at the rabbit colony

Of 38 rabbits serologically examined, 71.1% (n=27) had anti-*E. cuniculi* antibodies, and 21.2% (n=8/38) showed clinical symptoms; 20 out of 30 clinically healthy rabbits were seropositive in this colony. In addition, five cases revealed infection with *E. cuniculi* pathologically, although one symptomatic case was seronegative (Case 3) and four cases were not tested serologically (Cases 1, 2, 10 and 13). In total, 32 out of 42 (76.2%) rabbits in the colony were found to be pathologically and/or serologically infected with *E. cuniculi*.

Epidemiology of encephalitozoonosis and biosecurity measures

Since the identification of *E. cuniculi* in the colony, isolation and elimination of the suspected rabbits based on the results of serological examination were carried out immediately. Nevertheless, encephalitozoonosis continued to occur sporadically until the 15 March 2001. During the surveillance period, 25 rabbits with any disorders were presented at the animal hospital, and 44.0% (11/25) were diagnosed as encephalitozoonosis. On the other hand, there were no findings

regarded as reflecting encephalitozoonosis in any other species of animals examined pathologically.

Since *E. cuniculi* can also infect other animal species including humans, finally all the remaining rabbits in the colony were slaughtered for public health, then all the equipment such as floors, cages and feeding devices were washed thoroughly, followed by sterilization of with a burner, 70% ethanol or boiled water. After a two-month interval, new founder rabbits, five Lop-eared, three Rexes and five mixed breed, which are considered to be resistant to this disease, were introduced from an *E. cuniculi*-free rabbitry. These founder rabbits were quarantined for a week and subjected to inspections. As a result of long-term epidemiological monitoring, 10 years after the outbreak, no repeat of the encephalitozoonosis outbreak has been seen at the zoo.

Epidemiologically, new rabbit introductions before the first occurrence of infection were surveyed retrospectively. Encephalitozoonosis had never been identified over the 30 years since the opening of the zoo. Four rabbits were purchased from a pet shop and newly introduced to the colony without quarantine at the zoo on April 1998 and 1999. Three in 1998 (Cases 11, 18 and 19; one was not examined) and two in 1999 (Cases 6 and 7; the other two were negative) were found to be infected with *E. cuniculi* pathologically. They were suspected as being the source of the infection.

For the staff education, the animal keepers were informed that *E. cuniculi* is a

potential zoonotic risk and were told to avoid direct contact with the urine of even healthy animals to prevent the infection spread. Hygiene measures such as hand and fingers-washing and the subsequent disinfection with 70% ethanol were thoroughly performed after cleaning works.

Discussion

This is the first report of an outbreak of encephalitozoonosis that occurred in a rabbit colony at a zoo where a wide variety of animals were kept and the public visited in large numbers, and of the subsequent biosecurity measures with serology-based testing and slaughter in Japan. The surveillance revealed that asymptomatic infection with *E. cuniculi* was widespread among apparently healthy rabbits in this colony. On the other hand, the disease was not found in any other species of animal kept in the same area within the zoo facility. In countries in which outbreaks have been reported, serology-based testing and slaughter have been implemented to eliminate *E. cuniculi* from industrial or laboratory-based rabbit colonies (Cox et al. 1977; Bywater and Kllett 1978; Boot et al. 1988). However, in Japan, such preventative methods have not been applied at sufficient intensity. In the present study, biosecurity measures were successfully accomplished with regard to animal hygiene and public health; in particular, staff education, epidemiological surveillance and “all-out and all-in” colony establishment were key factors for the eradication of *E. cuniculi*.

Isolation of *E. cuniculi* organisms was attempted from an infected rabbit showing neurological symptoms (Case 14) using primary tissue culture techniques of the kidneys according to procedures reported previously (Furuya et al. 1995; 2001). *E. cuniculi* spores were successfully isolated from the collected sample, identified as *E. cuniculi* by PCR with a species-specific primer set and by direct DNA sequencing of the PCR products, and then registered as 2008FF in the GenBank (Furuya et al. 2001). The isolate was found to belong to genotype I on the basis of the number of 5'-GTTT-3' repeats in the internal transcribed spacer (ITS) of the ribosomal ribonucleic acid (RNA) genes (Furuya 2002).

The dwarf-breed rabbits that were purchased from a pet shop and newly introduced into the colony were considered as carriers of *E. cuniculi*, because the pet shops imported rabbits from the United States and Europe, where high seroprevalence of *E. cuniculi* has been reported (Greenstein et al. 1991; Keeble 2001; Hacourt-Brown and Holloway 2003). Moreover, the pure-blooded dwarf-breeds tended to be more easily affected by clinical symptoms than the others, suggesting that these breeds are predisposed to encephalitozoonosis, as reported previously (Kunstýr and Naumann 1985; Künzel and Joachim 2010).

In this study, 11 cases showed typical clinical symptoms such as central nervous dysfunction and occasionally fatal prognosis attributed to the encephalitozoonosis (Valencakova et al. 2008; Jeklova et al. 2010; Künzel and Joachim 2010). It was interesting that juvenile rabbits showed nonspecific symptoms such as weight loss

and stunted growth, finally leading to death. Therefore, encephalitozoonosis should be suspected and differentiated from other diseases in rabbits showing nonspecific and chronic clinical symptoms.

The specific and typical tissue lesions in infected rabbits were the same as those reported previously (Shadduck and Pakes 1971; Kunstýr and Naumann 1985; Levkut et al. 1998). The results of serological diagnosis almost correlated with the pathological results. Therefore, the effectiveness of ELISA and WB used in this study for biosecurity measures was confirmed. Antibody detection by serological examination indicates previous exposure to *E. cuniculi*. It was reported that the presence of IgM antibodies was considered indicative of active infection, even in asymptomatic rabbits (Jeklova et al. 2010). In this study, *E. cuniculi* organisms were more likely to be detected in the cases that were IgM antibody-positive and pathologically exhibited glial nodules with the focus of necrosis in the brain. A single seronegative case was found among the cases infected with *E. cuniculi* by pathological examination, which might be considered to reflect an early stage of infection, weak humoral immune response or degeneration of antibodies. Based on these results, all of the suspected rabbits in the colony were decided to be eliminated in order to eradicate the *E. cuniculi*, as opposed to individual care.

On the other hand, in rabbits that showed sudden onset of torticollis, the organisms were often found within the brain tissue, which is indicative of active infection. Although severe coccidia infection was also found in one case, it was not

observed in the other rabbits. This meant that multiple infections with another pathogen were not always necessary to trigger encephalitozoonosis in this study.

An *E. cuniculi* strain obtained from AIDS patients was classified as type I, which is the same as the rabbit strain detected in the present study, and which is supposed to be an opportunistic zoonotic pathogen (Didier et al. 1995; Deplazes et al. 1996; Rossi et al. 1998; Mathis et al. 2005). The same genotype of *E. cuniculi* has also been detected among both animal keepers and captive chimpanzees in several zoos, suggesting human-animal transmission (Sak et al. 2011). The zoo keepers work with the infected rabbits and also a wide variety of animal species. Moreover, people with weak immunity such as babies or the elderly visit the zoo to enjoy the experience of touching the animals. Therefore, eradication of this pathogen from the rabbit colony was considered necessary as a public health and animal hygiene measure. Firstly, only every rabbit suspected of being infected was isolated and eliminated; however, eradication of *E. cuniculi* was not achieved. Therefore, all the existing rabbits were finally slaughtered. Then, the facility was closed, and all the equipment was disinfected with a burner, 70% ethanol or boiled water, which has been confirmed to be effective against spores of *E. cuniculi* (Waller 1979; Jordan et al. 2006; Künzel and Joachim 2010). Fortunately, no animal keepers have shown any symptoms related to *E. cuniculi* infection during the surveillance period, although they have not been serologically tested for antibodies against *E. cuniculi*.

Greenstein *et al.* (1991) reported that a significant decline in the incidence of *E. cuniculi* infection in a commercial barrier-maintained rabbit colony was most likely due to a selection process for the breeding program instituted by the supplier, which was based upon the productivity, posture and weight of each animal. In order to establish a healthy rabbit colony, the importance of these selective factors, including breed, was emphasized and the introduction of individuals with suspected infection, such as those presenting with growth abnormality, was avoided. In the present study, after new colony establishment by the introduction of breeds that are considered to be resistant to this disease from *E. cuniculi*-free rabbitries, no repeat of this outbreak has occurred over 10 years.

In this study, serological examination has not been applied for the various other species of animal kept at the zoo. The natural infectivity of *E. cuniculi* against other animal species remains unclear. In the future study, it might be meaningful to obtain a deeper understanding of *E. cuniculi* ecology to reveal the prevalence of subclinical infection by detection of specific antibodies against *E. cuniculi* in a variety of animals at a zoo facility.

In conclusion, the results of the present study show the importance of biosecurity measures for preventing the spread of *E. cuniculi* infection, especially considering the potential zoonotic risk. If seropositive rabbits are present, application of an "all-out and all-in" system for rabbit colony establishment based on serological examination with disinfection of the animal-housing facility and an adequate

period without rabbit habitation should be successful for eradication of *E. cuniculi*. Rabbits are popular animals, kept not only by individual owners but also in zoos or schools for the purpose of providing children with opportunities to interact with these animals. Therefore, it should be considered necessary to apply biosecurity measures, including serodiagnostic examination, against *E. cuniculi* in order to eradicate it from rabbits owned by zoos, schools or for import quarantine in Japan

Summary

An outbreak of encephalitozoonosis occurred in a rabbit colony at a zoo in Japan. Throughout the two years after the onset, all 42 rabbits were investigated clinically, pathologically and serologically for the prevention and control of the disease. Eleven rabbits (11/42, 26.2%) showed clinical symptoms. Of 38 rabbits examined to detect specific antibodies against *Encephalitozoon cuniculi*, 71.1% (n=27) were found seropositive; 20 out of 30 clinically healthy rabbits (except for 8 clinical cases) were seropositive. The infection rate was 76.2% (32/42), including five pathologically diagnosed cases. The results of serological survey revealed that asymptomatic infection was widespread, even among clinically healthy rabbits. However, encephalitozoonosis was not found in any other species of animals kept in the same area within the zoo by pathological examination. Isolation and elimination of the rabbits with suspected infection based on the results of serological examination were carried out immediately; however,

encephalitozoonosis continued to occur sporadically. Therefore, all the remaining rabbits were finally slaughtered, and then, the facility was closed, and all the equipment was disinfected. After a two-month interval, founder rabbits were introduced from *E. cuniculi*-free rabbitries for new colony formation. Since then, encephalitozoonosis has not been seen in any animals at the zoo. In this study, biosecurity measures including staff education, epidemiological surveillance and application of an "all-out and all-in" system for rabbit colony establishment based on serological examination were successfully accomplished with regard to animal hygiene and public health for the eradication of *E. cuniculi*.

Table 3-1. Results of the clinical, serological and pathological examinations in 21 rabbits (*Oryctolagus cuniculus*) diagnosed as *E. cuniculi* infection.

Case	Breed	Age	Clinical findings	Serological diagnosis[a]			Pathological findings[b]		
				ELISA (IgG)	ELISA (IgM)	WB	Organism detection	Main lesion	Involved organs[c]
1	Angora lop[d]	40 days	Depression, stunted growth, ataxia, death	NT	NT	NT	3+	Glial nodule in brain 2+	K, CNS
2	Angora lop	40 days	Depression, stunted growth, ataxia, death	NT	NT	NT	3+	Glial nodule in brain 3+	K, CNS, H, Lu, Li
3	Angora lop	2.5 months	Depression, stunted growth, ataxia	—	—	—	3+	Glial nodule in brain 3+	K, CNS, Lu
4	Angora lop	2.5 months	None	+	+	3+	2+	Glial nodule in brain 2+	K, CNS, H, Lu, Li, S
5	Angora lop	2.5 months	None	+	+	3+	2+	Glial nodule in brain 2+	K, CNS, Lu, Li, S
6	Angora lop	> 1 year	Depression	+	+	3+	1+	Granulomatous encephalitis 2+	K, CNS, Lu, Li, S
7	Lop	> 1 year	None	+	—	3+	—	Granulomatous encephalitis 2+	K, CNS, Lu, Li, S
8	Lion lop	3 months	Depression, Severe torticollis, ataxia, hind-limb asthenia	NT	NT	3+	1+	Granulomatous encephalitis 2+	K, CNS, H, Lu, Li, S
9	Netherland dwarf	50 days	Depression, Light torticollis, stunted growth, ataxia	+	+	1+	2+	Granulomatous encephalitis 3+	K, CNS, Lu, Li, S
10	Lion lop	1 year / 1 year and 10 months	Light torticollis, ataxia, hind-limb asthenia / death	NT	NT	NT	1+	Granulomatous encephalitis 2+	K, CNS, H, Lu, Li, S
11	Angora lop	> 1.5 years	Depression, stunted growth	+	+	3+	1+	Granulomatous encephalitis 1+	K, CNS, H, Lu, Li, S
12	Lion	> 3.5 years	Depression, head shaking	+	+	3+	1+	Granulomatous encephalitis 2+	K, CNS, H, Lu, Li, S
13	Lion lop	4 months	None	NT	NT	NT	1+	Granulomatous encephalitis 2+	K, CNS, Lu, S
14	Lion-Angora mixed	3.5 years	Depression, Severe torticollis	+	—	3+	1+	Granulomatous encephalitis 2+	K, CNS, H, Lu, Li
15	Lion	2 years	Severe torticollis, ataxia, hind limb-asthenia, death	+	—	3+	—	Kidney infarctus 2+	K, CNS
16	Lop	> 3.5 years	None	+	+	2+	1+	Glial nodule in brain 1+	K, CNS, H, Lu, Li
17	Netherland dwarf	> 4 years	None	+	—	3+	—	Granulomatous encephalitis 1+	K, CNS, H, Li, S
18	Lion lop	> 3 years	None	+	+	3+	—	Glial nodule in brain 1+	K, CNS, H, Lu, Li, S
19	Lion lop	> 3 years	None	NT	NT	3+	1+	Granulomatous encephalitis 2+	K, CNS, H, Lu, Li, S
20	Lion	> 3 years	None	+	—	3+	—	Granulomatous encephalitis 2+	K, CNS, H, Lu, Li, S
21	Lion	10 months	None	+	+	3+	1+	Granulomatous encephalitis 2+	K, CNS, H, Lu, Li, S

[a]Serological diagnosis: NT=not tested; ELISA=enzyme-linked immunosorbent assay; antibody values are expressed as optical density, 1.0 or more was regarded as positive.

WB: western blot assay, a positive judgment was evaluated according to the presence of band formation, and 1+, 2+ and 3+ scores were defined depending on the number of bands.

[b]Detection of *E. cuniculi* organisms and main lesion: (3+)=severe; (2+)=intermediate; (1+)=mild.

[c]K=kidney; CNS=central nervous system; H=heart; Lu=lung; Li=liver; S=spleen.

[d]lop=lop-eared rabbit.

Fig. 3-1. Rabbits (*Oryctolagus cuniculus*) infected with *E. cuniculi*.
The rabbit on the left (Case 3) was found to have stunted growth compared with the rabbit of the same age on the right (Case 5).

CONCLUSION

In the present thesis, the author summarized clinical, pathological and epidemiological findings of three incidents of emerging infectious diseases (EIDs) between wildlife and captive animals in a zoo, of which little is known about the prevalence in Japan. The first records were documented on 1) a mass mortality in Eurasian Tree Sparrows (*Passer montanus*) caused by *Salmonella* Typhimurium DT40 resulting in the population decline and the detection of *S.* Typhimurium DT120 in Japan, 2) an emergence of novel avian pox in Carrion Crows (*Corvus corone*) and a Large-billed Crows (*C. macrorhynchos*) and the outbreak in the Japanese avifauna, and 3) an outbreak of encephalitozoonosis in domestic rabbits (*Oryctolagus cuniculus*) housed in a rabbit colony at a zoo, with the subsequent biosecurity measures by using serology-based testing. In addition, improved diagnostic protocols for the early detection and identification of the wildlife EIDs, and control measures of these diseases were proposed. For the early and accurate diagnosis, the first in important was to detect characteristic clinical symptoms of the diseases based on understanding of 'normal' animal behaviors as a baseline, and to investigate the patients using a combination of several diagnostic methods. Furthermore, this study focused on a potential role of zoo veterinarians in advanced health management of valuable animal collections in captivity including monitoring and biosecurity measure for wildlife EIDs, which can contribute to *in*

situ conservation of wildlife as well as to *ex situ* conservation.

The present study revealed the following three points associated with the epidemiology of the wildlife EIDs between wildlife and captive animals in a zoo and zoo biosecurity measures.

In chapter 1, it was demonstrated that an emerging epidemic infection with *S.* Typhimurium DT40 related to bird feeding was the cause of sparrow mortality in 2008–09 in Asahikawa area. The mass mortality caused decline of the local sparrow population. The first case found on a zoo ground showed unusual clinical symptoms such as weakness, inability to fly, hypothermia, and died 2 hr later, although such captured wild sparrows are relatively uncommon in winter. The sparrow was investigated to evaluate the cause of death by a zoo veterinarian as a preventive medicine program for biosecurity. Consequently, the small wild bird provided us "early warning" signals to detect this avian EID that caused the following mass mortality in sparrows. Since the identification of this unusual emergence of wildlife disease, *Salmonella* screening survey of the zoo captive animals was conducted and they were confirmed negative. Long-term epidemiological monitoring of salmonellosis using tree sparrows as sentinels is important to evaluate the further impact on sparrow populations, livestock hygiene and public health.

In chapter 2, the present study demonstrated that an epizootic of emerging avian pox occurred in crows, which might have led to mortality of the juveniles in

Hokkaido, 2006–10. Since the first identification of avian pox in a crow in Sapporo, 2006, the mortality incidents peaked in 2007–08, resulted in subsequent spread out different Hokkaido areas. In Sapporo, the outbreak was considered to have negatively impacted on the local crow population. Interestingly, the sequence detected in a zoo-kept crow seemed to be independent from any reported clades, and thus interspecies transmission was suspected. As a zoo biosecurity measure, health management practices were conducted to prevent the disease spread to captive birds in the nearby cages (i.e., isolation of the patient and screening of captive birds). Furthermore, avipoxviruses and crows can be useful as an epidemiological model of zoonotic arboviruses including West Nile virus, transmitted among wildlife hosts by arthropod vectors.

In chapter 3, an outbreak of emerging encephalitozoonosis occurred in a zoo rabbit colony was described. The results of serological survey revealed high prevalence of *Encephalitozoon cuniculi* infection (71.1%) and widespread asymptomatic infection in the colony. *E. cuniculi* infection has not been determined in any other species of animals kept in the same area within the zoo by routine clinical and pathological examinations as epidemiological monitoring for 10 years after the outbreak, although encephalitozoonosis is considered as an important EID in both wildlife and humans. To establish an *E. cuniculi*-free rabbit colony, biosecurity measure including staff education, epidemiological surveillance and application of an "all-out and all-in" system based on serological examination was successfully

accomplished. In zoos, unusual disease transmission may occur among species (in zoo animals or between exotic species and native species in the wild) in unnatural environment; however, it can occur even in nature in the near future, possibly enhanced by global climate change or increased international movements of animals and humans. Thus, it should be important to record unusual clinical cases by interspecies transmission and even negative data of epidemiological surveillance during a disease outbreak, providing information of host susceptibility.

In conclusion, the present study demonstrated the integrated diagnostic protocols for the wildlife EIDs, especially, the first importance of understanding in animal normal behaviors and unusual clinical symptoms, and routine clinical and pathological examination in diseased animals including complete necropsies on every dead animal on a zoo ground. In addition, potential effective function of zoos was proposed as a novel data source for biosurveillance of wildlife EIDs and zoonoses, and as a wildlife health center. Moreover, this thesis presents captive wildlife and zoo animals in the close association with humans, especially common species (i.e., sparrows, crows and rabbits) can also serve as important sentinels for the EIDs. In the near future, this study could contribute to encourage zoos and professional zoo veterinarians to develop an effectively integrated surveillance system of wildlife EIDs in the collaboration with human and animal health organizations, which should contribute to ecological health.

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SUMMARY IN JAPANESE

本論文では、野生動物と動物園飼育動物に共通に感染しうる新興感染症（EIDs）について、国内ではその発生と分布がほとんどわかっていない疾病の発生 3 事例の臨床、病理および疫学的所見をまとめた。以下に、新しく得られた知見の概要を示す。1）スズメ（*Passer montanus*）の個体数減少を引き起こした *Salmonella* Typhimurium DT40 感染症による集団死と *S.* Typhimurium DT120 の国内初記録、2）ハシボソガラス（*Corvus corone*）とハシブトガラス（*C. macrorhynchos*）における鳥ポックスウイルス（APV）の新興感染と流行（＝国内鳥類相における APV 感染症の集団発生の初記録）、3）動物園のカイウサギ（*Oryctolagus cuniculus*）飼育群において集団発生したエンセファリトゾーン症に対して国内で初めて血清学的診断法を用いて制御に奏功したバイオセキュリティ対策、である。これらの EIDs の正確な早期診断には、第一に、基準値として動物種ごとの“正常な”行動生態を理解した上で、疾病ごとの特徴的な臨床症状に気づき、複数の診断手法を組み合わせて鑑別診断することが重要であった。加えて、動物園獣医師の役割として、動物園の貴重な飼育個体群に対する質の高い健康管理における EIDs のモニタリングおよびバイオセキュリティ対策を通じ、野生動物の生息域外保全のみならず、生息域内保全に貢献し得ることを整理して示した。

第 1 章では、旭川地域において 2008～2009 年に発生したスズメの集団死の原因は、*S.* Typhimurium DT40 感染症の新興感染と餌台に起因した流行によるものと明らかにした。この集団発生は、スズメの地域個体群の個体数減少を引き起こした。初

発例は、スズメがあまり保護されることのない冬に、動物園の敷地内で保護され、衰弱、飛翔不能および低体温症などの不自然な臨床症状を示して死亡した。そのため、著者は動物園獣医師として、飼育動物のバイオセキュリティ対策のため、予防医学プログラムの一環として、そのスズメの死因の検索を行った。結果として、この 1 羽の野鳥の異常死は、その後、スズメの地域個体群で集団死が発生することの前兆となった。また、この不自然な野生動物感染症の発生を受け、動物園飼育動物のバイオセキュリティ対策として、サルモネラのスクリーニング検査を実施し、陰性を確認した。

第 2 章では、北海道のカラス類において、2006～2010 年に流行した幼鳥に致死的な APV の新興感染を明らかにした。カラス類の APV 感染症による死亡事例は、2006 年に札幌で初発例の確認後、2007～2008 年をピークとして増加し、道内の他地域でも発生し、APV 感染の拡大が考えられた。札幌のカラス類における集団発生は、地域個体群の減少を引き起こしたと考えられた。興味深いことに、動物園で飼育されていたハシブトガラスから検出された APV の P4b 遺伝子配列は、DDBJ データベース上に登録はなく、これまで報告されているいずれの系統からも独立しており、異種間感染が示唆された。感染拡大防止のため、感染個体の隔離や近接する鳥類飼育施設のスクリーニング検査などバイオセキュリティ対策を実施した。

第 3 章では、一動物園のウサギ飼育施設におけるエンセファリトゾーンの新興感染と浸淫状況を明らかにした。血清学的調査を実施し、本飼育個体群の *Encephalitozoon cuniculi* の高い抗体保有率（71.1%）と不顕性感染個体の存在が明らかとなった。本症は、野生動物やヒトの重要な EID となっていることから、同じエリア内の飼育施設の他の動物種について、日常的な臨床学的検索および死亡個体の病理学的検索を実

施し、10 年間に渡ってモニタリングしたが、発生は認めなかった。バイオセキュリティ対策として、スタッフの教育、疫学調査および血清学的検査に基づくウサギ飼育個体群の全淘汰後に創設個体の新規導入を行い、新たな *E. cuniculi* フリーのウサギ飼育個体群の形成に成功した。多種多様な動物種が飼育されている動物園では、人工的な飼育環境下で、“不自然な”感染症の伝播が起こりうるが、近い将来、気候変動や動物・人の移動などの要因が関わって、このような感染症が自然界でも実際に発生する可能性がある。そのため、動物園において、異種間感染のまれな臨床例、あるいは感染症の流行時に疫学調査を実施して得られた結果を例え陰性結果でも記録して蓄積しておくことは、宿主感受性がわかるなどの有益な情報になりうると考えられる。

本論文は、野生動物と動物園飼育動物に共通に感染しうる 3 つの EIDs の総合的な診断プロトコールについて、特に、動物種ごとの正常な行動生態の理解に基づく特徴的な臨床症状の早期検出および動物園の敷地内で死亡あるいは異常を示す飼育動物と野生動物の日常の病理検査の重要性を明らかにした。また、本研究は、動物園が野生動物の EIDs や人と動物の共通感染症に関する豊富な情報を有する感染症のサーベイランス拠点および保全医学的視点を備えたワイルドライフヘルスセンターとして機能する可能性を提示した。さらに、人に身近な野生動物および動物園飼育動物、中でもスズメ、カラスやウサギといった普通種は、野生動物の EIDs を早期発見するために歩哨動物として機能しうるということが示され、重要な視点である。近い将来、我が国でも動物園や動物園獣医師が野生動物の EIDs の統合的サーベイランスのために人や動物の衛生研究機関と協働して効果的に機能することに役立ち、健全な生態系の保全に貢献することができると考える。