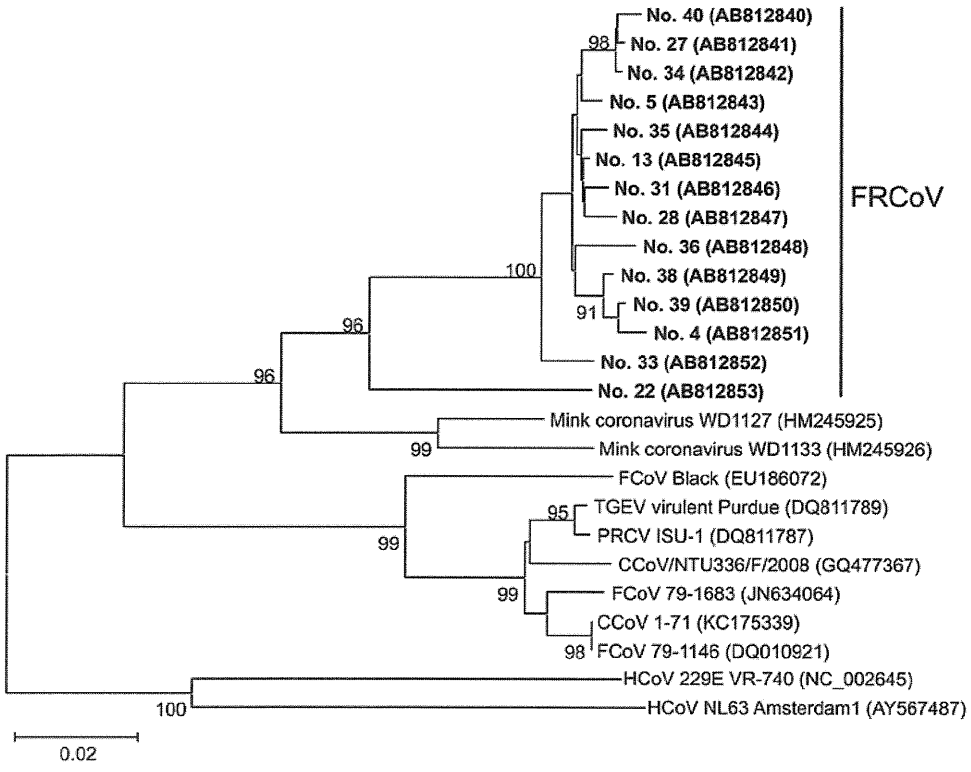

Figure 1. Phylogenetic tree constructed on the basis of the nucleotide sequences of the partial RNA-dependent RNA polymerase–encoding regions of ferret coronaviruses (FRCoVs) isolated in Japan (shown in boldface; sample IDs are indicated) compared with other coronaviruses (CoVs). The tree was constructed by the neighbor-joining method in MEGA5.0 software (*10*); bootstrap values of >90 are shown. DDBJ/EMBL-Bank/GenBank accession numbers for the nucleotide sequences are shown in parentheses. Human CoVs (HCoVs) 229E and NL63, which belong to the *Alphacoronavirus* genus, were used as the outgroup. CCoV, canine coronavirus; FCoV, feline coronavirus; TGEV, transmissible gastroenteritis virus; PRCoV, porcine respiratory coronavirus. Scale bar indicates nucleotide substitutions per site.

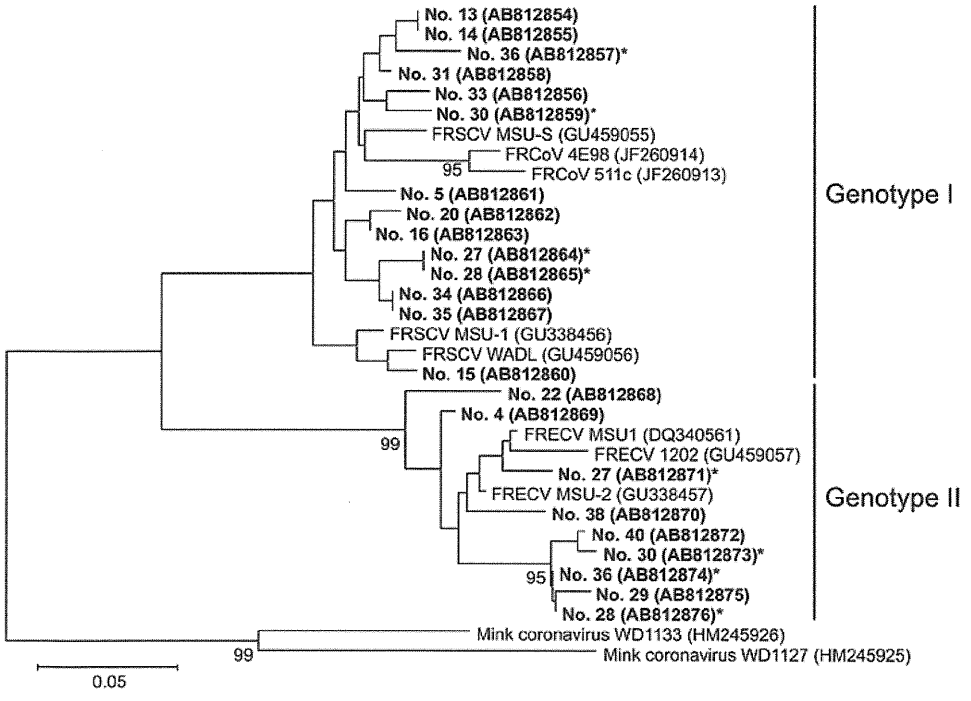

Figure 2. Phylogenetic tree based on the nucleotide sequences of partial S genes of ferret coronaviruses (FRCoVs) isolated in Japan (shown in boldface; sample IDs are indicated) compared with other coronaviruses (CoVs). The tree was constructed by the neighbor-joining method in MEGA5.0 software (*10*); bootstrap values of >90 are shown. Asterisks indicate samples from ferrets infected with FRCoVs of both genotypes1 and 2. DDBJ/EMBL-Bank/GenBank accession numbers for the nucleotide sequences are shown in parentheses. FRSCV, ferret systemic coronavirus; FRECV, ferret enteric coronavirus. Scale bar indicates nucleotide substitutions per site.

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MASS MORTALITY OF EURASIAN TREE SPARROWS (*PASSER MONTANUS*) FROM *SALMONELLA* TYPHIMURIUM DT40 IN JAPAN, WINTER 2008–09

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ABSTRACT: 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. We conducted a comprehensive investigation to evaluate the cause and impact on sparrow populations. We collected 26 carcasses at 13 sites, including a zoological park. In addition, *Salmonella* screening of zoo animals was conducted as a biosecurity measure. *Salmonella* 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 zoo animals. Isolates belonged to three phage types: DT40 (88%), DT110 (8%), and DT120 (4%). Pulsed-field gel electrophoresis patterns were the same in all isolates, regardless of phage type. 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, and 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. We conclude that an emerging epidemic infection with *S.* Typhimurium DT40 related to bird feeding was the cause of sparrow mortality in 2008–09 and suggest that this causative strain is host-adapted to sparrows in Japan. The mortality might have had some impact on the local population, but its influence was limited.

Key words: Ecological health, epidemiology, Eurasian Tree Sparrows, humans, mass mortality, *Salmonella* Typhimurium DT40, salmonellosis, wild bird feeding.

INTRODUCTION

Epizootics of salmonellosis in passerines caused by *Salmonella enterica* subsp. *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 emerging infectious disease of wild birds because its prevalence appears to have increased over the past 40 yr (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 event 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 (D.F. unpubl. data), or *Staphylococcus aureus* infection (M.A. unpubl. data), 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 zoological park. We conducted a comprehensive investigation and diagnosed salmonellosis due to *S.* Typhimurium DT40 as the cause of mortality. A *Salmonella* screening survey in zoo animals was conducted for animal hygiene and public health because association of *S.* Typhimurium infection in zoo birds with House Sparrow salmonellosis was also reported (Alley et al. 2002; Rabsch et al. 2002). We report epidemiologic data and field survey results, evaluate the influence of this mortality on Eurasian Tree Sparrow populations, and discuss zoo biosecurity countermeasures.

MATERIALS AND METHODS

Mortality reports and specimens

In winter 2008–09, multiple Eurasian Tree Sparrow deaths occurred around Asahikawa, Hokkaido, Northern 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 Asahiyama Zoological Park, was presented to the zoo animal hospital on 11 January 2009 and immediately died. We performed postmortem examination for a preventive medicine program.

Finally, 202 deaths in 100 incidents at 94 sites were reported to the Kamikawa Subprefectural Office of Hokkaido Government by private citizens 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).

Epidemiologic field surveys

We conducted epidemiologic field surveys at the 13 sites. We collected sparrow information from residents and directly observed 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). Questionnaires focused on dates and numbers of carcasses found, duration of incidents, location, presence of feeding sites, 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 microbiologic 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 virologic, microbiologic, toxicologic, and histopathologic examinations. Tracheal

TABLE 1. Summary data of postmortem findings of 26 carcasses of Eurasian Tree Sparrows (*Passer montanus*) diagnosed as salmonellosis caused by *Salmonella* Typhimurium in Japan, 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 January	A	+[d]	+	Y	20.6	+	−	+/NA	+/NA	−/NE	+/NA	+	DT40
2	Prior to 10 January	A	+[d]	+	Y	18.9	+	+	+/NA	−/NE	+/+[e]	+/NA	+	DT40
3	10 January	A	+[d]	+	Y	20.1	+	+	+/NA	+/−	+/−	+/NA	−	DT40
4	10 January	A	+[d]	+	Y	18.6	+	+	+/+[e]	−/NE	+/+	−/−	+	DT40
5	11 January	A	+[d]	+	Y	18.6	+	+	+/+[e]	+/+[e]	−/−	+/NA	−	DT40
6	11 January	B[f]	+[d]	+	Y	21.1	+	+	+/+[e]	+/−	−/NE	+/−	−	DT40
7	6 February	B[f]	+[d]	+	Y	22.3	−	−	+/+	+/+	−/−	+/NA	−	DT40
8	7 February	C	+[d]	−	Y	17.3	+	−	+/+	−/+	+/+	+/−	+	DT40
9	8 February	D	+	+	A	22.0	−	−	+/+	+/+	−/−	+/NA	+	DT40
10	11 February	E	+[d]	+	Y	19.1	+	−	+/+	+/+	−/+	+/NA	+	DT40
11[g]	16 February	F	−	NE	A	21.3	+	−	+/NA	+/−	+/+	+/NA	−	DT40
12[h]	16 February	F	−	NE	Y	16.7	+	+	−/NA	−/NA	+/+	+/NA	−	DT120
13	Prior to 24 February	G	+[d]	+	Y	21.0	+	+	+/NE	−/NE	−/NE	NA/NE	+	DT40
14	Prior to 24 February	G	+	+	U	19.4	+	+	+/NE	−/NE	+/NE	−/NE	+	DT40
15	24 February	G	+	+	A	19.3	+	+	+/+	−/−	+/+	+/NA	+	DT40
16	23 February	H	+[d]	+	Y	18.7	+	+	+/+	+/+	+/−	+/NE	+	DT40
17	28 February	H	+	+	Y	17.7	+	+	−/NA	−/+	+/NE	+/NA	+	DT110
18[g]	1 March	H	+	+	A	16.0	+	−	+/+	+/+	−/−	+/−	−	DT40
19	1 March	H	+	+	A	21.7	+	+	+/+	−/+	+/NE	+/NA	−	DT40
20	3 March	I	+	−	A	18.9	+	+	−/+	−/−	+/NE	+/NA	−	DT110
21	3 March	I	+	−	A	20.1	+	−	+/+	+/+	+/−	+/NA	+	DT40
22	7 March	J	+	NE	A	17.7	+	+	+/NA	−/−	−/−	+/NA	+	DT40
23	12 April	K	+	NE	U	NE	+	−	NA/NA	NA/NA	+/NA	NA/NA	NA	contaminated
24	10 April	L	−	NE	U	NE	+	NA	NA/NA	NA/NA	NA/NA	NA/NA	NA	DT40
25	28 April	M	+	NE	U	NE	+	−	NA/NA	NA/NA	NA/NA	NA/NA	NA	DT40
26	28 April	M	+	NE	U	NE	+	−	NA/NA	NA/NA	NA/NA	NA/NA	NA	DT40

TABLE 1. Continued.

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]	
% positive or mean values		13 sites	88.0%[i]	75% (6/8)	Y: 62% A: 38%	19.4 g	92%	56%	82%/ 100%	50%/ 63%	61%/ 47%	91%/ 0%	59%	DT40: 88% DT110: 8% DT120: 4%

[a] ST = *Salmonella enterica* subspecies *enterica* serovar Typhimurium; DT = definitive phage types; Y = young; A = adult; U = uncertain; 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 (A, B, C, E, G, and H) 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.

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

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

and cloacal swabs were obtained for virologic examination for avian influenza virus (Sakai-Tagawa et al. 2010) and West Nile virus (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 pathologic 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. The body weights were compared to those of 11 healthy wild Eurasian Tree Sparrows captured from January to March 2010 with permission from the Hokkaido Government. Systematic external and internal examinations of body systems were performed and any gross lesions were recorded.

Swab samples with Transwab 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.

Bacteriologic examination

All collected swab specimens were plated onto a selective medium for isolation. *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).

In *S.* Typhimurium isolates, antimicrobial susceptibility testing was done according to the standards outlined by the Clinical and Laboratory Standards Institute (2005) with the following drugs: ampicillin, amoxicillin, piperacillin, amoxicillin-clavulanate, cefazolin, ceftazidime, ofloxacin, trimethoprim-sulfamethoxazole, 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, DNA of the isolates was digested using infrequently cutting restriction enzymes *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 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 (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

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. These included the Japanese Cranes, Oriental Turtle Doves, domestic fowl (*Gallus gallus domesticus*), Pekin ducks (*Anas platyrhynchos domestica*), European rabbits (*Oryctolagus cuniculus*), guinea pigs (*Cavia porcellus*), domestic dogs (*Canis lupus familiaris*), timber wolves (*Canis lupus* ssp.), Hokkaido brown bears (*Ursus arctos yesoensis*), polar bears (*Ursus maritimus*), lions (*Panthera leo*), Amur tigers (*Panthera tigris altaica*), Amur leopards (*Panthera pardus orientalis*), black leopards (*Panthera pardus* var.), snow leopards (*Panthera 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*), Northern 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 and site B was the crane aviary within the Asahiyama zoo where multiple sparrow deaths had occurred.

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 the software JMP version 8.02 (SAS Institute, Cary, North Carolina, USA).

RESULTS

Epidemiology

February was the peak month for the number of birds found dead (Fig. 1). Using general information on the 26

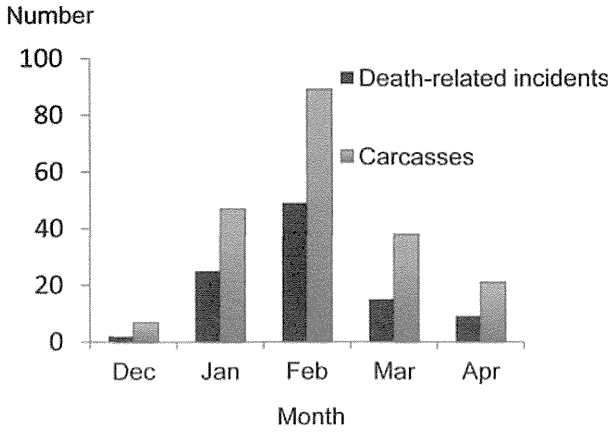

FIGURE 1. Number of death-related incidents and carcasses of Eurasian Tree Sparrows (*Passer montanus*) by month, reported during a period of mass mortality due to salmonellosis in Hokkaido, Northern Japan, winter 2008–09. Between December 2008 and April 2009, residents reported 202 sparrow deaths in 100 incidents at 94 sites.

carcasses as well as gross/histopathologic findings and results of virologic and bacteriologic tests (Table 1), all examined dead sparrows were diagnosed with 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, fecal samples were *S.* Typhimurium-positive.

Clinical signs

The initial case within the zoo showed weakness, lethargy, inability to fly, and hypothermia and died after 2 hr. 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 pathologic findings in sparrow carcasses

At necropsy, carcasses showed various degrees of tissue damage from autolysis. In the intestines of 19 cases (73%), carcass decomposition prevented histopathologic 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 ($n=10$), congestion in the spleen ($n=11$), splenomegaly ($n=7$), ingluvitis ($n=14$), enteritis ($n=19$), and hemorrhage within skull air spaces ($n=13$). 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).

The distribution of histopathologic lesions is shown in Table 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 colonies of bacteria 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 avian influenza and West Nile viruses.

Bacteriologic findings

Salmonella Typhimurium was isolated from all examined organs in all necropsied sparrows. All isolates showed a very weak reaction in a 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. 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, apart from one case with contamination: 88% DT40 (22/25), 8% DT110 (2/25), and 4% DT120 (1/25).

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 2). Calcium concentrations in brain and liver in salmonellosis cases were significantly lower than those of normal birds ($P<0.05$).

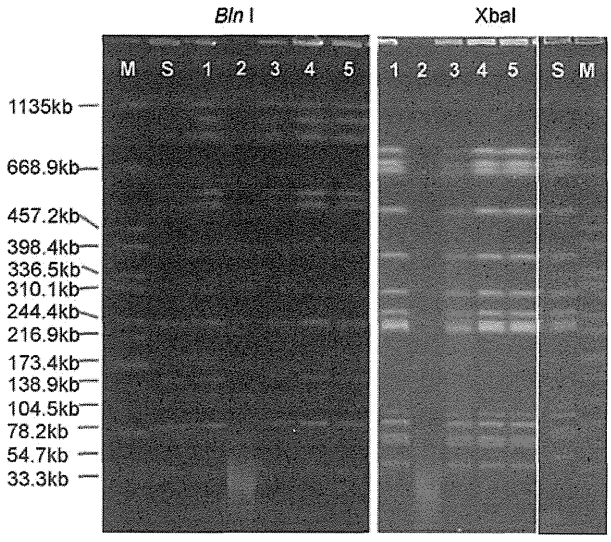

FIGURE 2. *Bln*I and *Xba*I-digested pulsed-field gel electrophoresis patterns of *Salmonella* Typhimurium isolated from Eurasian Tree Sparrow (*Passer montanus*) samples in Hokkaido, Japan, winter 2008–09. M = a molecular size marker (S. Braenderup H9812 digested with *Bln*I and *Xba*I, respectively); 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.

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

Changes of population numbers in sparrows monitored at two sites are shown in Figure 3. Dead sparrows with salmonellosis were collected near sites A and B: six (Cases 16–21) and two carcasses (Cases 6 and 7), respectively. Few sparrows were seen between April and October. 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 the next winter, it recovered in 2010–2011 (data not shown).

TABLE 2. Concentrations of sodium (Na), calcium (Ca), and magnesium (Mg) in brain, liver, and muscle obtained from salmonellosis Eurasian Tree Sparrows (*Passer montanus*), Hokaido, Japan, 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 examined	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 the salmonellosis group.

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

DISCUSSION

Septicemic salmonellosis was the main cause of mass mortality in Eurasian Tree Sparrows in Japan during winter 2008–09. Bird feeding sites contaminated by *S.* Typhimurium-positive droppings (e.g., on and beneath tables and sites where food was scattered) can be important sources for exposure to *Salmonella*. This supports previous reports that 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).

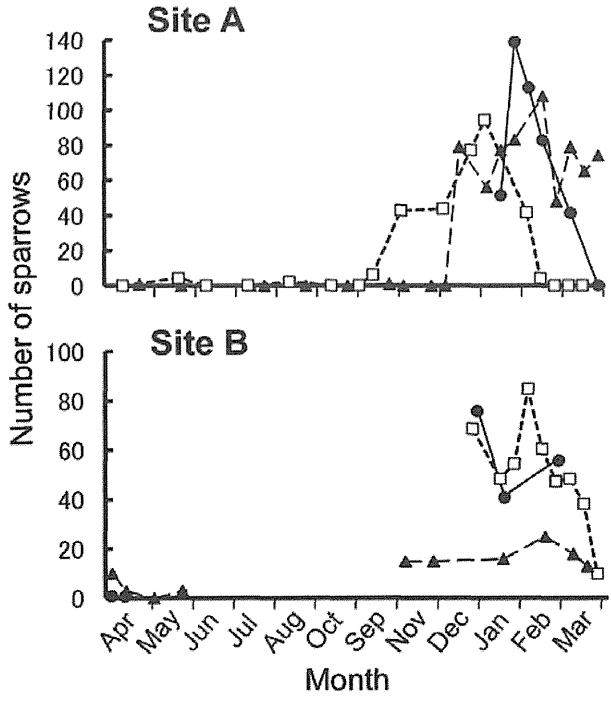

FIGURE 3. Changes in population abundance of Eurasian Tree Sparrows (*Passer montanus*) counted in 2007–10 at two monitoring sites related to wild bird feeding where carcasses were found during a period of mass mortality due to salmonellosis in Japan, winter 2008–09. Site A was near the town of Nishikagura, Site B was a crane aviary in the Asahiyama zoo. —●—2007–08; ···□···2008–09; - -▲- - 2009–10.

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 con-

tact rates; Hudson et al. 2000; Tizard 2004). In addition, immature sparrows in their first winter may be more affected because of weak 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 *S.* Typhimurium phage type we isolated 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 (Y.U. unpubl. data).

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 a 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 to 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. Our findings differed from a previous report describing house sparrows with *S.* Typhimurium DT160 infection having a 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 immunologic 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). We observed the disappearance of sparrows 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 needed to evaluate the potential influence of salmonellosis on sparrow populations.

The die-off incidents of sparrows in 2005–06 and 2008–09 in Hokkaido occurred primarily in Asahikawa and Sapporo, the two largest cities. This might relate to the expansion of the human population and increased urbanization, leading to reduced wildlife habitat and increased congregation of sparrows at feeding sites in urban areas in winter, as reported in the US (Hall and Saito 2008). These conditions may increase contact among sparrows and with other wildlife species, humans, and domestic animals, increasing the risk for 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 sparrow mortality period in 2005–06, *S.* Typhimurium DT40 was first recorded in Japan in 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. *Salmonella* Typhimurium DT40 has not been detected 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 spill over 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 Central Asian 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 only isolated 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), we found no evidence of sparrow ingestion of chemical deicer. The reason for significantly lower calcium levels in brain and liver in salmonellosis sparrows was unclear. We should determine the normal calcium level in healthy sparrows because values also varied among control samples, as the SD indicated.

After we recognized the initial salmonellosis case in the zoo, we immediately conducted *Salmonella* screening of zoo animals and found all samples negative. Epidemiologic 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.

Epidemic infection with *S.* Typhimurium DT40 caused mass mortality of Eurasian Tree Sparrows around Asahikawa, Japan, in winter 2008–09 in association with anthropogenic feeding of birds. For ecologic 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, 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 control the disease.

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Experimental Infection of Mongolian Gerbils with *Baylisascaris potosis*

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?1 ABSTRACT: The present study evaluated the pathogenicity of *Baylisascaris potosis*, a newly described ascarid nematode, in Mongolian gerbils. Gerbils were infected with varying doses of either *B. potosis* or *Baylisascaris transfuga* embryonated eggs (100, 1,000, and 4,000) for 30 days postinfection (pi). *Baylisascaris potosis*–infected gerbils showed no clinical signs of disease; however, gerbils exposed to 1,000 and 4,000 *B. transfuga* eggs showed severe neurologic signs at 22–29 days and 14–15 days pi, respectively. Histopathologic examination revealed larvae and lesions in the intestine, lung, liver, and muscles of *B. potosis*–infected gerbils, but not in the brain, whereas *B. transfuga* larvae were found only in the brain and muscle. These results indicate that *B. potosis* larvae migrate through numerous organs and are associated with visceral larva migrans in gerbils, but less frequently migrate to the nervous system in gerbils than does *B. transfuga*.

Baylisascaris is an ascarid nematode genus whose members are primarily found in carnivores and are often implicated in larva migrans (LM) in a number of wildlife, domestic animals, and humans. In 2013, *Baylisascaris potosis*, the kinkajou roundworm, was discovered in captive kinkajous (*Potos flavus*) imported from the Co-operative Republic of Guyana (Tokiwa et al., 2014). Morphologic and molecular analyses demonstrated that *B. potosis* closely resembles *Baylisascaris procyonis*, a cause of zoonotic visceral, ocular, and severe cases of neural LM, but information about the pathogenicity of *B. potosis* is lacking.

Kinkajous are nocturnal mammals native to tropical forests of South and Central America. Although kinkajous are rising in popularity as exotic pets, the Centers for Diseases Control and Prevention (CDC) reported that *Baylisascaris* infections may occur in pet kinkajous and warned about the risk of human exposure to this parasite from animals (Kazacos et al., 2011). Therefore, it is important to examine the infectivity and pathogenicity of *B. potosis* isolated from kinkajous. In the present study, we examined the clinical and histopathologic characteristics of LM caused by experimental *B. potosis* infection in Mongolian gerbils (*Meriones unguiculatus*); Mongolian gerbil is reportedly the best animal model of neural LM caused by *B. procyonis* and *Baylisascaris transfuga* ?2 (Sato et al., 2004; Cho et al., 2009).

Mongolian gerbils (n = 16) aged between 5 and 6 wk were raised in our laboratory and maintained under pathogen-free conditions. Fertile *B. potisis* and *B. transfuga* eggs were collected from captive kinkajou and Asian black bear (*Ursus thibetanus*) feces, incubated for 1 mo at 27 C to produce embryonated eggs, and stored at 4 C until needed. The embryonated eggs for infection were estimated by counting the eggs containing motile larvae per unit volume. The eggs were inoculated into the stomach of each gerbil under light anesthesia using a metal gastric probe. The gerbils were randomly divided into 6 groups. Three groups (G1, G2, and G3) comprising 3 subjects each, were inoculated with *B. potosis* eggs, and the other 3 groups (G4, n = 2; G5, n = 3; and G6, n = 2) were inoculated with *B. transfuga* eggs. G1 and G4 were inoculated with 100 eggs; G2 and G5 with 1,000 eggs; and G3 and G6 with 4,000 eggs. Infected gerbils were observed daily for the onset of clinical signs and were sacrificed if any sign of deteriorated condition was observed, i.e., rotating movement, continuous circling, severe ataxia, or lateral recumbence. Gerbils without clinical signs of disease were sacrificed by cervical fracture 30 days postinfection (pi). The brain, heart, spleen, lung, intestinal wall, liver, kidney, eye, and femoral muscles were fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections 3-μm in thickness were stained with hematoxylin and eosin. This animal experiment was approved by the Animal Experiments Ethics Committee of Azabu University (130207-3).

In the groups infected with *B. potosis* and in G4, there were no clinical signs during the observation period. In G5, loss of vigorous prostration was seen at 20–26 days pi, and infected gerbils showed severe ataxia and leaning at 22–29 days pi. In G6, vigorous prostration was lost at 11–12 days pi, and all gerbils showed severe ataxia, leaning, and/or rotating movement by 15 days pi.

Macroscopically, the *B. potosis*–infected gerbils, except for those in G1, showed multiple and white nodules in the subserosal tissue of intestine (Fig. 1A), intercostal muscles, diaphragm, heart, and liver. No abnormalities were seen in the brains of the *B. potosis*–infected gerbils. One subject in G6 showed only a few hepatic nodules. No nodules were detected in the other *B. transfuga*–infected gerbils. Histopathologically, *B. potosis* larvae measured 62.5–67.5 μm in diameter (Fig. 1E) and were found in the intestines (Fig. 1B), liver (Fig. 1C), heart, and muscle (Fig. 1D, E) of G2 and G3 gerbils; the *B. transfuga* larvae were found only in the brain and muscle of G5 and G6 gerbils. Both larval populations appeared nondegenerate but were surrounded by or embedded in granulomatus tissue (Fig. 1B–D). Numerous hemosiderin deposits were found in the lungs of G2, G3, G5, and G6 gerbils, and a few hemosiderin deposits were found in the livers of G5 and G6 gerbils. No histologic lesions were found in any of the ocular sections.

Sato et al. (2004) reported that *B. procyonis* LM was fatal to mice and gerbils, whereas *B. transfuga* LM was fatal only in gerbils but not in mice, and the *B. procyonis* larval lesions were much more severe than lesions induced by *B. transfuga* larvae in the brain of gerbils. Cho et al. (2007) suggested that *B. procyonis* larvae in gerbils were more likely to accumulate in the brain compared with *B. transfuga* larvae, and the severity of neural LM could be attributed to the total amount of larvae in the brain. In the present study, *B. potosis* larvae, and associated lesions, localized in several organs, but they did not occur in the brain. These findings suggest that *B. potosis* larvae can cause visceral LM in rodents and that the larval migration behavior to the brain in gerbils differs from that of *B. transfuga* and *B. procyonis*. Future studies are needed to fully characterize the migration behavior and pathogenicity of *B. potosis* in captive and wild animals, as well as in humans. The morphologic features and dimensions of *B. potosis* larvae within tissue closely resemble those of related species in the *Baylisascaris* genus, and it is difficult to differentiate from one another. Molecular biology techniques characterizing ribosomal and mitochondrial DNA sequences (Taira et al., 2013; Tokiwa et al., 2014) could prove useful in measuring *B. potosis* prevalence in captive and wild animals.

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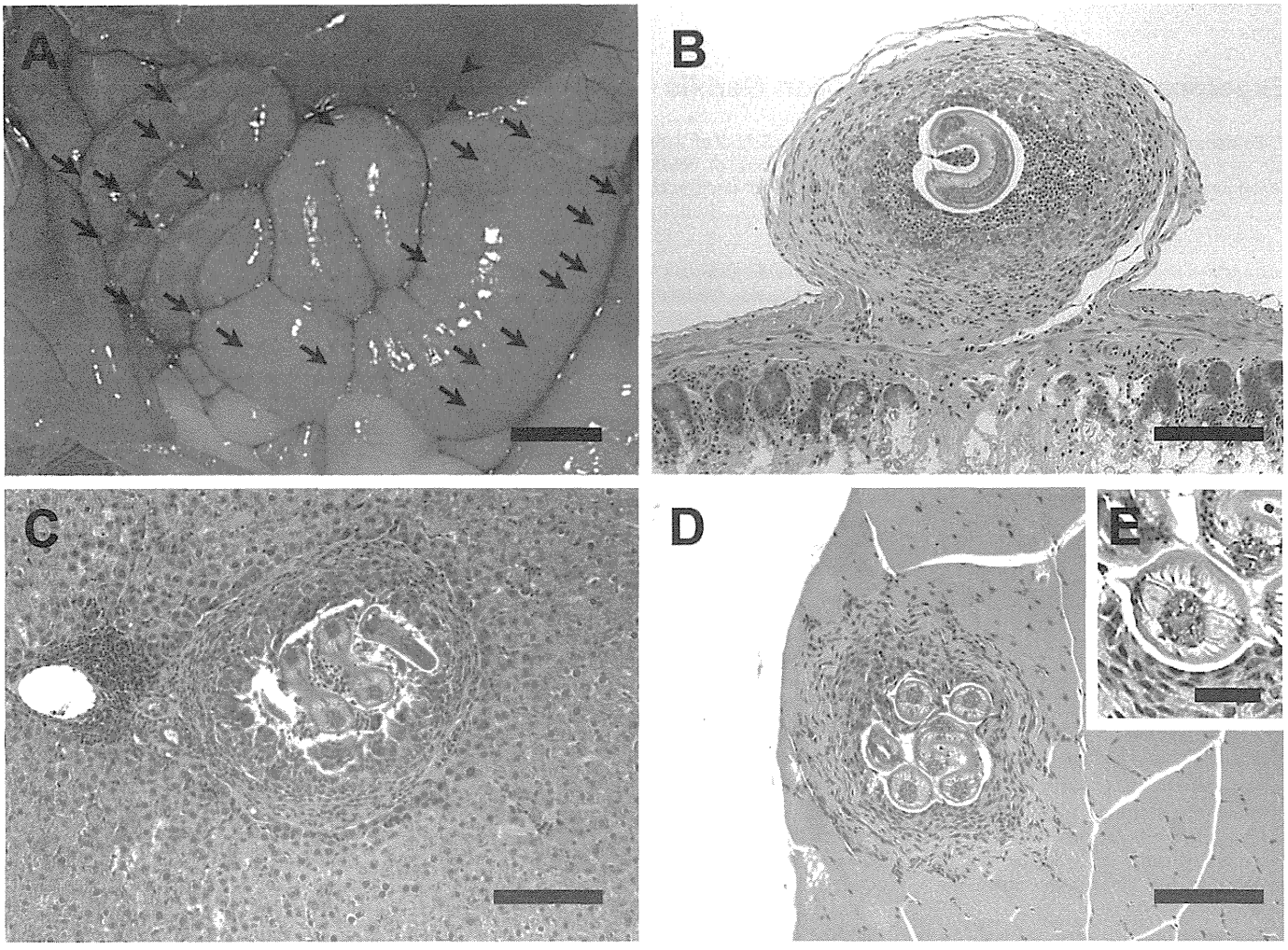

FIGURE 1. *Baylisascaris potosis* larvae in infected gerbils at 30 days postinfection. (**A**) Multiple white nodules are disseminated in the subserosal tissue of the intestine (arrows) and liver (arrowheads); scale bar = 5 mm. (**B**) Intestine; scale bar = 150 μm. (**C**) Liver; scale bar = 150 μm. (**D**) Muscle; scale bar = 150 μm. (**E**) Magnified transverse section of larva within the muscle; scale bar = 30 μm.

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Case report

The first report of peritoneal tetrathyridiosis in squirrel monkey (*Saimiri sciureus*)

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ABSTRACT

This report describes a case of peritoneal larval cestodiasis caused by tetrathyridia of *Mesocestoides* sp. in an adult female squirrel monkey. The monkey had lived in a zoological garden in Japan and had a clinical history of wasting. At necropsy, numerous whitish oval masses were found in the liver and peritoneal cavity. These masses contained larval cestodes. Morphological observation and molecular analyses of the mitochondrial 12S ribosomal RNA gene and cytochrome c oxidase subunit 1 gene sequences allowed us to identify the larva as the tetrathyridium of *Mesocestoides* sp. This is the first report of *Mesocestoides* larvae in a squirrel monkey in Japan.

1. Introduction

The adult cestode of the genus *Mesocestoides* (Cestoda: Cyclophyllidea) is usually found in the small intestine of carnivores and rarely human beings. The tetrathyridium, the second larval stage of the *Mesocestoides*, is found in various organs and the body cavity of various animals [1]. Due to the absence of specific morphological characteristics of tetrathyridia of the *Mesocestoides* genus, identification at the species level based on morphological observation is extremely difficult [2,3]. However, molecular tools have been developed for the reliable identification of *Mesocestoides* [4,5]. Here, we report a case of peritoneal larval cestodiasis and the identification of these cestodes isolated from a squirrel monkey (*Saimiri sciureus*) in Japan. Our findings represent the first case report of tetrathyridium infection in squirrel monkeys.

2. Case report

In February 2013, one adult (>5 years old) female squirrel monkey that was bred in a zoological garden in Kyusyu region of Japan died suddenly. At the time of death, the monkey was wasting and showed hypotrichosis of the limbs, tail, and abdomen, and its weight was 450 g.

At the post-mortem examination, numerous soft whitish masses, 1.19 ± 0.1 mm (0.9–1.4) (mean ± S.D. (minimum–maximum); $n = 10$) in diameter, were found in the peritoneal cavity (Fig. 1A). The liver was slightly enlarged and had a yellow-brown appearance, and multiple oval-shaped masses were present in both lobes of the liver and were well circumscribed in tissues (Fig. 1B). Yellow serpentine tracts approximately 2 to 8 mm long were observed on the diaphragmatic surface of the left lateral lobe and visceral surface of the left median and gradate lobes (Fig. 1B). Microscopically, masses in the peritoneal cavity and liver contained one or two motile cestode larvae (Fig. 1C) which had numerous small calcareous corpuscles.

Histopathologically, the invaginated scolex of the cestode larvae had four well-developed suckers and was unarmed with hooks (Fig. 1D). Staining by von Kossa's method revealed numerous calcareous corpuscles in the body of the larvae. Mild infiltration of lymphocytes surrounded the larvae in the liver tissues (Fig. 1D). A severe degree of degeneration, disarray of hepatic chords, and diffuse vacuolar degeneration of hepatocytes were observed in the liver tissues. The remaining hepatocytes often showed multifocal necrosis with coagulative necrosis or mitotic alternations. In addition, a section of encapsulated rictulariid nematode larvae was observed in the liver tissues. Other findings were emphysema in the lung tissues and diffuse myocardial degeneration.

Total DNA of the cestode larvae was extracted using a QIAamp DNA Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The forward primer P60F (5′-TTAAGATATATGTGGTTACAGGATTAGATACCC-3′) and the reverse primer P375R (5′-AACCGAGGGTGACGGGCGGTGTGTACC-3′) [6] were used for mitochondrial 12S ribosomal RNA (12S) gene amplification. The primers Cyclo_cox1Fa (5′-CARCATATGTTTTGRTTTTTTGG-3′) and Cyclo_cox1Rb (5′-CCTAAYGACATAACATAATGRAAATG-3′) described by Littlewood et al. [7] were used to amplify the

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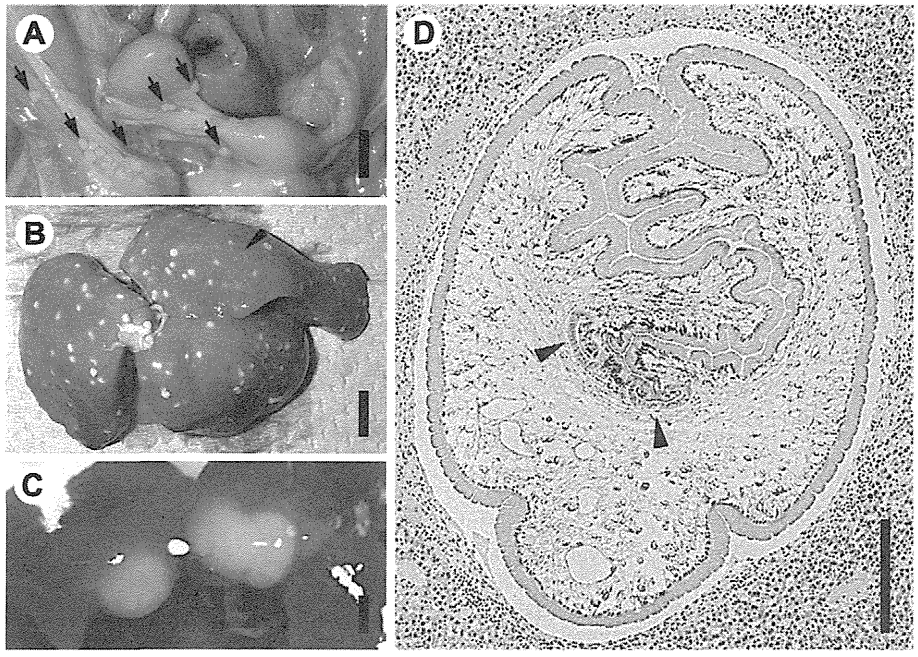

Fig. 1. Larval cestodes in the squirrel monkey. (A) Numerous whitish masses (arrows) were found in the peritoneal cavity. *Bar* = 0.5 cm; (B) diaphragmatic surface of the liver showing the numerous whitish masses and a serpentine tract (arrowhead). *Bar* = 0.5 cm; (C) tetrathyridia at a high magnification. *Bar* = 0.5 mm; (D) an encapsulated tetrathyridium with invaginated scolex and suckers (arrowheads) in the liver. *Bar* = 300 μm.

mitochondrial cytochrome c oxidase subunit 1 (*cox1*) gene. The PCR products were purified and directly sequenced on both strands. A 283-bp of 12S and 412-bp of *cox1* sequences were obtained and were deposited at the DDBJ database under accession number AB908164 (12S) and AB932596 (*cox1*), respectively. The BLAST search demonstrated that the 12S sequence of the larvae from the squirrel monkey was 86–91% compatible with the reference sequence of *Mesocestoides* species published in the GenBank/DDBJ/EMBL databases. The *cox1* sequence shared 89% identity with *Mesocestoides lineatus* (GenBank/DDBJ/EMBL accession no. AB792715). Neighbor-joining phylogenetic tree inferred from 12S sequence using MEGA 6.0 demonstrated that the *Mesocestoides* isolated from the squirrel monkey and *M. lineatus* formed a monophyletic group with high bootstrap values (Fig. 2). The 12S sequence obtained in this study differed from that of *M. lineatus* with *p*-distances of 0.090–0.127, from *Mesocestoides corti* (syn. *Mesocestoides vogae*) including *Mesocestoides* sp. B with *p*-distances of 0.090–0.125, from *Mesocestoides* sp. A with *p*-distances of 0.129–0.131, from *Mesocestoides* sp. C with *p*-distances of 0.130–0.145, and from *Mesocestoides litteratus* with *p*-distances of 0.125–0.131.

3. Discussion

Based on morphological characteristics and molecular analyses, the cestode larvae were identified as *Mesocestoides* tetrathyridia. The

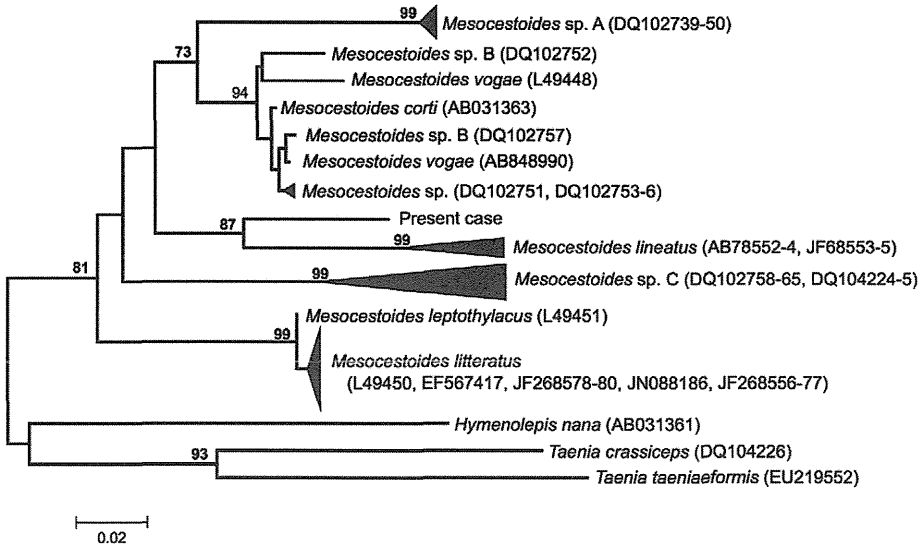

Fig. 2. Phylogenetic tree based on 12S sequences of *Mesocestoides* spp., showing the position of the isolates from the squirrel monkey (present study). Numbers at nodes represent bootstrap values (>70%) for 2000 replicates.

occurrence of tetrathyridium in non-human primates is rare, having only been reported in baboons [8], macaques [9,10], vervet monkeys [11], and white-throated monkeys [12]. To our knowledge, this is the first report of tetrathyridium in squirrel monkeys.

Phylogenetic analyses based on the sequence of the second internal-transcribed spacer 2 revealed at least 3 distinct lineages within *Mesocestoides* parasitizing canids in North America [13]. Another phylogenetic study using 12S supported these 3 lineages (clades A, B, and C) and confirmed that *M. lineatus*, *M. litteratus*, and *Mesocestoides* spp. belonging to different clades are separate species [5,14]. In Japan, *M. lineatus* was reported in domestic dogs [15], *Mesocestoides paucitesticulus* in raccoon dogs, the Japanese red fox and the Japanese marten [16–18], *M. corti* (syn. *M. vogae*) in dogs [19], and unidentified *Mesocestoides* spp. in mongooses and the tsushima leopard [20]. Our molecular analyses revealed that the *Mesocestoides* tetrathyridium isolated in this study is clearly different from *M. lineatus* and *M. corti* (syn. *M. vogae*). Unfortunately, we could not compare our sequence with *M. paucitesticulus*, the species reported in Japan, because there are no available DNA sequences of *M. paucitesticulus* in the databases.

The life cycle of the members of the genus *Mesocestoides* is not precisely defined, but, insects can serve as the first intermediate hosts [21,22]. Various mammals, birds, reptiles, and amphibians can serve as second intermediate hosts. These hosts acquire the infection by eating the insects containing cysticercoid larvae. After ingestion, these larvae penetrate through the small intestinal wall, the abdominal cavity, and the liver parenchyma, and develop into an infective tetrathyridium. The tetrathyridium observed in this case indicated that squirrel monkeys could serve as second intermediate hosts.

In the present case, monkeys bred at the zoological garden were free feeding and eat insects as a part of their natural diet. Even though the source of the infection in this case remains unknown, it was most likely the result of feeding on infected first intermediate hosts containing larvae. However, almost nothing is known about the range of intermediate hosts of the members of the genus *Mesocestoides* in natural conditions in Japan. In conclusion, this is the first case of tetrathyridium infection in a captive squirrel monkey. The taxonomy and biology of the genus *Mesocestoides* remains unsolved. Therefore, further studies are needed to better assess the identification of the species, and their transmission route.

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