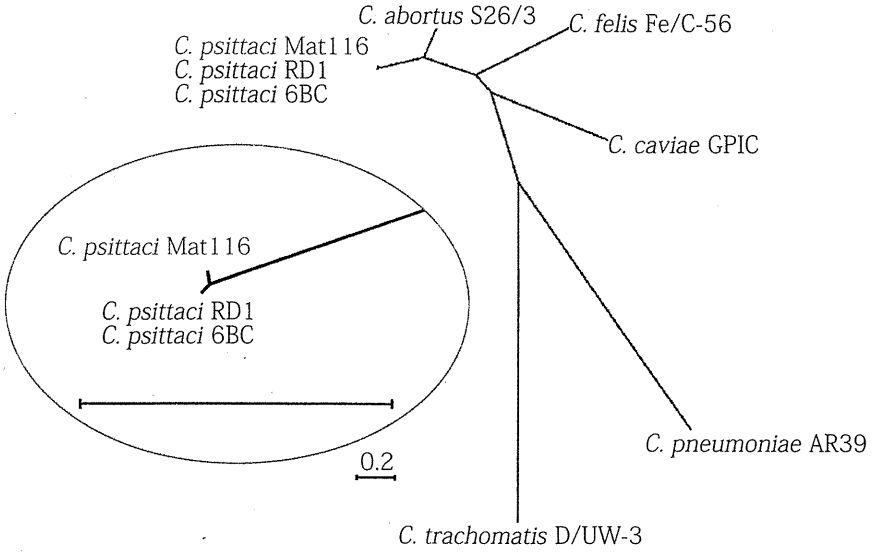

各種クラミジアについて，4種類のハウスキーピング遺伝子（*enoA*，*gidA*，*gatA*，*hlfX*）を連結後アライメントし，MEGA ver. 5.02を用いて解析した。系統樹はNJ法にて作製した。遺伝距離は0.2 units barで示す。

図1　4種類のハウスキーピング遺伝子を用いたクラミジア種間の系統解析

株との詳細な比較解析により，本菌の人獣共通感染症としての多様な宿主域や病態に関するヒントが得られるかも知れない。現在は，他種クラミジアとの比較解析の結果同定した，*C. psittaci* 特異的遺伝子を標的とした鑑別診断法を開発中である。また由来や病原性の異なる複数株の配列解読も進行中である。以上のようなアプローチを通じて，オウム病クラミジアの生態の理解，制御に少しでも貢献できればと考えている。

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NOTE *Virology*

Molecular Genetic and Pathogenic Characterization of Psittacid Herpesvirus Type 1 Isolated from a Captive Galah (*Eolophus roseicapillus*) in Japan

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ABSTRACT. Psittacid herpesvirus type 1 (PsHV-1) was isolated from a captive galah (*Eolophus roseicapillus*) in Japan that was suspected of having Pacheco's disease (PD), an acute fatal disease in psittacine birds. PsHV-1 has been classified into four genotypes based on the UL16 gene sequence. In the present study, we investigated the genetic and pathogenic characteristics of the isolated virus, FOY-1, compared with a reference strain, RSL-1. The FOY-1 strain was classified into PsHV-1 genotype 2. The FOY-1 strain was found to be less pathogenic to budgerigars than RSL-1, which was classified as genotype 4 in an *in vivo* study. This is the first report regarding the classification of originally isolated PsHV-1 in Japan and its characterization by animal infection experiment.

KEY WORDS: genotype, pathogenicity, psittacid herpesvirus.

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To date, psittacid herpesvirus (PsHV) is the only herpesvirus in psittacine birds, and the virus has been classified as an avian member of the *Alphaherpesvirinae* subfamily by the International Committee on Taxonomy of Viruses [11]. PsHV type 1 (PsHV-1) is the causative agent of Pacheco's disease (PD), an acute fatal disease in psittacine birds [9]. Outbreaks of PD have resulted in massive die-offs of infected birds. Recently, another type of psittacid herpesvirus, PsHV-2, was identified in three African grey parrots (*Psittacus erithacus*) [10]. The sequences of the UL16, whose putative function is capsid assembly, and UL30, whose putative function is DNA polymerase, regions of PsHV-2 both differ from the most closely related PsHV-1 by more than 20%. However, the prevalence and pathogenicity of PsHV-2 is unclear [10, 14].

Based on the UL16 gene sequence, PsHV-1 has been classified into four genotypes. All four of the PsHV-1 genotypes have the potential to cause PD but have distinct biological characteristics [13]. It has been reported that the susceptibility to each PsHV-1 genotype depends on the bird species [13]. For instance, Amazon parrots (*Amazona* spp.) are the most common parrot species diagnosed with PD, and all four genotypes of PsHV-1 have been isolated from these species. PsHV-1s of all four genotypes were also found among birds from the Pacific region such as cockatiels (*Nymphicus hollandicus*) and cockatoos (*Cacatua* spp.). On the other hand, PsHV-1s from African grey parrots with PD have been classified as genotypes 2, 3 and 4, but not genotype 1. In the case of macaws (*Ara* spp.) and conures (*Aratinga* spp. and *Pyrrhura* spp.), genotype 4 has been commonly found and causes mortality, genotype 3 has rarely been found and is less pathogenic and genotypes 1 and 2 have not caused PD at all.

PsHV-1 infections have been reported in several species of psittacine birds in many countries including the U.S.A. [6, 9], U.K. [2], Spain [1] and South Africa [4]; however, genetic and pathogenic information about PsHV-1 is limited. In Japan, avian herpesvirus has been detected in psittacine birds by electron microscopy [15]. However, the virological investigation has been limited. This communication describes the genetic characteristics of PsHV-1 isolated from a captive galah (*Eolophus roseicapillus*) in Japan. In addition, the pathogenicity of this isolate was evaluated by experimental infections using budgerigars (*Melopsittacus undulatus*), a psittacine species that is easier to use than other bird species.

A galah died after exhibiting anorexia and diarrhea in a bird sanctuary in Japan in 2002. Pathological and microbiological examinations were performed in this study to investigate the cause of death. At necropsy, hepatomegaly and splenomegaly were observed (data not shown). Histopathological examination revealed multifocal necrosis in the liver and spleen (data not shown). Intranuclear inclusion bodies were observed in hepatocytes in the liver (Fig. 1) and macrophages in the spleen (data not shown). PCR was carried out in order to detect common pathogens in psittacine birds including herpesvirus [16], beak and feather disease (BFDV) [17] and avian polyomavirus (APV) [7]. DNA was extracted from 50 mg of liver and spleen samples with a SepaGene nucleic acid extraction kit (Sanko Junyaku Co., Tokyo, Japan) according to the manufacturer's instructions. As a result, herpesviral DNA, but no DNA of other pathogenic viruses, was detected in both the liver and spleen (data not shown).

A primary culture of chicken embryo fibroblast (CEF)

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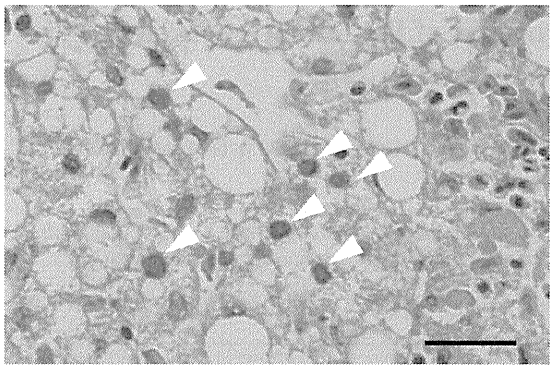

Fig. 1. Histological section of the liver of the galah. Eosinophilic intranuclear inclusion bodies were found in the hepatocytes. The typical inclusion bodies are indicated by arrowheads. Hematoxylin and eosin stain; bar=25 μm.

was used to isolate the herpesvirus. The monolayer was inoculated with 10% (w/v) homogenate of liver or spleen in cell culture medium at 37°C for 1 hr. The inoculated cultures were washed twice with cell culture medium and incubated at 37°C. The cultures were observed for cytopathic effect (CPE) daily. CEF cells inoculated with the liver and spleen samples showed a CPE 2 and 4 days after inoculation, respectively. The CPE was characterized by ballooning and detachment of the cells from the cell culture plate. After three passages, the presence of herpesviral DNA in the culture was confirmed by the PCR used above at 2 days postinoculation. The virus isolated was designated as FOY-1.

The PCR used above was broadly applicable to the detection of the DNA polymerase coding region of herpesviruses in humans and animals [16]. Next, the PCR amplification pattern was examined using five primer sets to investigate further genetic characteristics of FOY-1 as described by Tomaszewski [12]. The locations of primer sets 9F, 9R, 11F, 11R and 23F were UL19 (major capsid protein), UL21 (nucleocapsid protein), UL15 (DNA packaging protein), UL9 (ori binding protein) and UL16, respectively. Amplicons of FOY-1 were produced by using primer sets 9F, 11F and 23F but not 9R and 11R (Fig. 2A). The RSL-1 strain (ATCC VR-915) [3], which is a reference strain for PsHV-1 in this study, was detected with all five primer sets. Tomaszewski *et al.* reported that ten patterns (PsHV-1 variants v1 to v10) were observed using these five primer sets [12]. Although RSL-1 was classified into PsHV-1 variant 1 in this study, the pattern of FOY-1 has never been reported. Since the 23F primer set was able to detect all PsHV-1 variants, FOY-1 was thought to be PsHV-1.

Since PCR amplification patterns of the PsHV-1 have been reported to partly indicate the genotype [13], next, the genotypes of FOY-1 and RSL-1 were identified. A 420-bp fragment of the UL16 gene was generated by PCR with the 23Ff5a/b primer set, which was used for genotyping of PsHV-1 strains in a previous study [10], and sequenced. Partial UL16 gene sequences of FOY-1 and RSL-1 were submitted to DDBJ (Accession numbers: AB510905 and AB510906). A putative 140-amino acid sequence was compared with each of the published PsHV-1 sequences shown in Table 1. The FOY-1 amino acid sequence was closest (99.2% identity) to that of the 1070/93 strain, which is grouped into PsHV-1 genotype 2. The FOY-1 sequence was 79.2% homologous to that of RSL-1.

In a phylogenetic tree based on the UL16 amino acid sequence and constructed by the UPGMA method with the Genetyx-Mac version 15.0.1 computer software (Fig. 2B), the PsHV-1 strains fell into four genotypes, in agreement with the results of Tomaszewski [13]. FOY-1 belongs to genotype 2, whereas RSL-1 belongs to genotype 4. Restriction fragment length polymorphism (RFLP) analysis of the entire viral genomic DNA with restriction endonucleases *Eco*RI, *Pst*I and *Bgl*II was performed to subdivide the genotypes [8]. According to the migration profiles, 12 different restriction patterns have been recognized [8]. FOY-1 was classified as an FFF virus, whereas RSL-1 was classified as an AA2A2 virus (Fig. 2B and 2C). The groups are named on the basis of the cleavage patterns with the restriction endonucleases *Eco*RI, *Pst*I and *Bgl*II. For instance, the first F of FFF and A of AA2A2 mean patterns obtained with *Eco*RI, the second F and A mean *Pst*I, and the third F and A mean *Bgl*II [8].

The pathogenicities of FOY-1 and RSL-1 (isolated from rosellas) were compared using budgerigars as an experimental host. All experimental infection plans were approved by the Committee for Animal Research and Welfare of Gifu University. Twenty-one conventional budgerigars were divided into seven groups and kept in isolators. Before the experiment, we checked whether the birds had been infected with any pathogens, such as herpesvirus, BFDV and APV, by the PCR described above. Three groups of three budgerigars each were orally inoculated with 0.1 m*l* of the FOY-1 strain with 10^6 $TCID_{50}$/bird (Group A), 10^4 $TCID_{50}$/bird (Group B) or 10^2 $TCID_{50}$/bird (Group C), respectively. The other three groups were inoculated with 0.1 m*l* of the RSL-1 strain with 10^6 $TCID_{50}$/bird (Group D), 10^4 $TCID_{50}$/bird (Group E) or 10^2 $TCID_{50}$/bird (Group F), respectively. The virus titers used for inoculation were calculated by a limiting dilution method taking into consideration the CPE as reported in a previous study [5]. The last group was inoculated with 0.1 m*l* of PBS and served as the control (Group G). The birds were observed daily for any clinical signs of disease. At 14 days postinoculation (d.p.i.), the surviving birds were euthanized with an intramuscular injection of ketamine hydrochloride. Liver and spleen samples of all the birds were collected for histopathological examination and herpesviral DNA detection. PCR was carried out using the five primer sets used above to confirm whether the inoculated virus was detected in their tissues [12].

One bird of group A (A-1) showed piloerection at 8 d.p.i., and two birds of group D (D-1 and D-2) showed anorexia and depression at 6 d.p.i (Table 2). No clinical signs were observed in the other birds. At 7 or 8 d.p.i., one bird spontaneously died in each RSL-1-inoculated group. The other

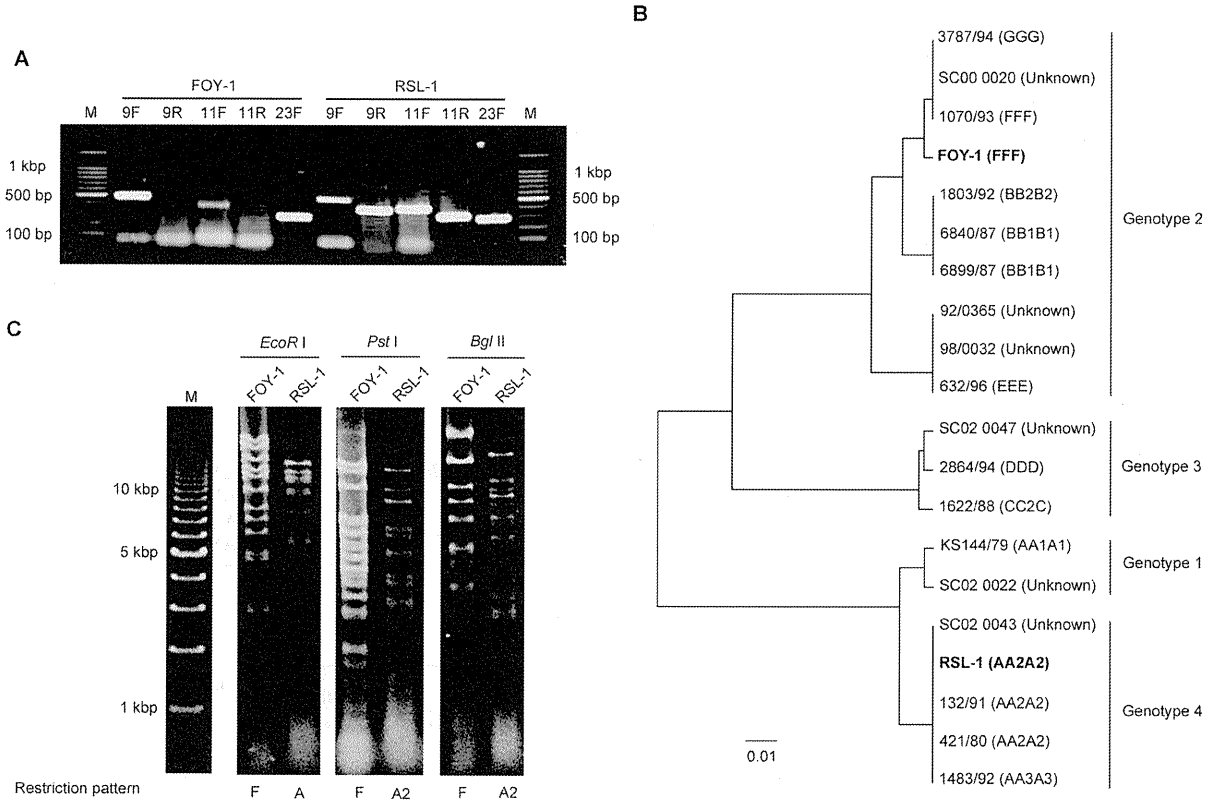

Fig. 2. Genetic characteristic of the PsHV-1 FOY-1 strain. (A) PCR amplification patterns of FOY-1 and RSL-1. Primer names (9F, 9R, 11F, 11R and 23F) according to Tomaszewski *et al.* are shown above each lane [12]. The M lane is a 100-bp DNA Ladder (ToYoBo, Osaka, Japan). (B) Phylogenetic analysis of FOY-1, RSL-1 and other reference strains based on the amino acid sequence of UL16. The tree was obtained using the UPGMA method with the Genetyx-Mac version 15.0.1 computer software. Restriction fragment length polymorphism (RFLP) patterns according to Schroder-Gravenduck *et al.* are shown in parentheses [8]. (C) RFLP patterns of FOY-1 and RSL-1 digestion with *Eco*RI, *Pst*I and *Bgl*II. Restriction patterns according to Schroder-Gravenduck *et al.* are shown under the photographs [8]. The M lane is a 1-kbp DNA Ladder (ToYoBo).

birds in the RSL-1-inoculated groups remained alive until the end of the experiment. On the other hand, all the birds in the FOY-1-inoculated groups remained alive. The overall mortality rate of the RSL-1-inoculated groups, i.e., total number of dead birds in the RSL-1 groups divided by total number of birds in the RSL-1 groups, was 33.3%, whereas the overall mortality rate of the FOY-1 groups was 0%. The histopathological changes in all three dead birds consisted of typical lesions of PD as reported by other researchers [9], such as coagulation necrosis in the liver and eosinophilic intranuclear inclusion bodies in hepatocytes and splenic reticular cells (data not shown). In addition, intranuclear inclusion bodies were found in hepatocytes of the two surviving birds of group D. A histopathological examination found no lesions in the tissues of the other surviving birds. Herpesviral DNA was detected in the liver of all the birds inoculated with RSL-1, whereas the rates of detection of viral DNA in the liver in the three FOY-1-inoculated groups (A, B and C) were 33.3, 0 and 33.3%, respectively.

In conclusion, a genotype 2 strain of PsHV-1 (FOY-1) was isolated from a captive galah that died after exhibiting anorexia and diarrhea and was less pathogenic to budgerigars than RSL-1 of genotype 4. This is the first report regarding the classification of originally isolated PsHV-1 in Japan and its characterization by an animal infection experiment.

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Table 1. PsHV-1 strains used for sequence analysis in this study

Genotype	Virus strain	Host	Restriction pattern	Accession no.
1	KS144/79	Blue-fronted Amazon (*Amazona aestiva*)	AA1A1	AY282614
	SC02–0022	Blue and gold macaw (*Ara ararauna*)		AY421993
2	6840/87	Blue-fronted Amazon (*Amazona aestiva*)	BB1B1	AY282624
	6899/87	African grey parrot (*Psittacus erithacus*)	BB1B1	AY282626
	1803/92	Pooled sample (*Amazons, pionus and conure*)	BB2B2	AY282627
	92/0365	Red-lored Amazon (*Amazona autumnalis*)		AY282631
	98/0032	Unknown		AY282634
	632/96	Green-cheeked Amazon (*Amazona viridigenalis*)	EEE	AY282630
	1070/93	Orange-winged Amazon (*Amazona amazonica*)	FFF	AY282628
	3787/94	White cockatoo (*Cacatua alba*)	GGG	AY282629
	SC00 0020	Yellow-headed Amazon (*Amazona oratrix*)		AY421988
3	1622/88	St. Lucia Amazon (*Amazona versicolor*)	CC2C	AY282635
	2864/94	St. Lucia Amazon (*Amazona versicolor*)	DDD	AY282640
	SC02 0047	Blue-fronted Amazon (*Amazona aestiva*)		AY421999
4	132/91	Yellow-crowned Amazon (*Amazona ochrocephala*)	AA2A2	AY282664
	421/80	African grey parrot (*Psittacus erithacus*)	AA2A2	AY282660
	1483/92	Cuba Amazon (*Amazona leucocephala*)	AA3A3	AY282668
	SC02 043	Blue and gold macaw (*Ara ararauna*)		AY421995

Genotype as presented in Tomaszewski *et al.* [11] and Styles *et al.* [8].

Table 2. Summary of the experimental infection design and results

Group	Virus strain	Dose ($TCID_{50}$/bird)	Bird no.	Clinical symptoms on each day postinoculation													Histopathological lesion	Detection of viral DNA in tissue sample	
				1	2	3	4	5	6	7	8	9	10	11	12	13		Liver	Spleen
A	FOY-1	10^6	1	–	–	–	–	–	–	–	+	–	–	–	–	–	–	+	+
			2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
			3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B	FOY-1	10^4	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
			2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
			3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
C	FOY-1	10^2	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+
			2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
			3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D	RSL-1	10^6	1	–	–	–	–	–	+	X							+	+	+
			2	–	–	–	–	–	+	–	–	–	–	–	–	–	+	+	–
			3	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–
E	RSL-1	10^4	1	–	–	–	–	–	–	–	X						+	+	+
			2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
			3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
F	RSL-1	10^2	1	–	–	–	–	–	–	X							+	+	+
			2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
			3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
G	PBS	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
			2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
			3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

A plus sign in the "Clinical symptoms on each day postinoculation" columns indicates that the bird showed clinical symptoms such as piloerection, anorexia, and depression on the indicated day. A minus sign indicates that no clinical symptoms were observed. An "X" indicates that the bird died on the indicated day. A plus sign in the "Histopathological lession" column indicates that typical lesions of PD, such as coagulation necrosis in the liver and eosinophilic intranuclear inclusion bodies, were observed by histological examination. A minus sign indicates that no lesions were observed. A plus sign in the "Detection of viral DNA in tissue sample" columns indicates that the viral DNA was detected in the tissue samples by PCR using the five set primers to confirm the inoculated virus strain. A minus sign indicates that no viral DNAs were detected.

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GENETIC CHARACTERISTICS AND ANTIMICROBIAL RESISTANCE OF *ESCHERICHIA COLI* FROM JAPANESE MACAQUES (*MACACA FUSCATA*) IN RURAL JAPAN

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ABSTRACT: *Escherichia coli* was isolated from wild and captive Japanese macaques (*Macaca fuscata*) to investigate the risk of zoonotic infections and the prevalence of antimicrobial-resistant *Escherichia coli* in the wild macaque population in Shimokita Peninsula, a rural area of Japan. We collected 265 fresh fecal samples from wild macaques and 20 samples from captive macaques in 2005 and 2006 for *E. coli* isolation. The predominant isolates were characterized by serotyping, virulence gene profiling, plasmid profiling, pulsed-field gel electrophoresis (PFGE), and microbial sensitivity tests. In total, 248 *E. coli* strains were isolated from 159 fecal samples from wild macaques, and 42 *E. coli* were isolated from 17 samples from captive macaques. None of the virulence genes *eae*, *stx*, *elt*, and *est* were detected in any of the isolates. The relatedness between wild- and captive-derived isolates was low by serotyping, PFGE, and plasmid profiling. Serotypes O8:H6, O8:H34, O8:H42, O8:HUT, O103:H27, O103:HNM, and OUT:H27 were found in wild macaque feces; serotypes O157:H42 and O119:H21 were recovered from captive macaques. O- and H-serotypes of the 26 isolates were not typed by commercial typing antisera and were named OUT and HUT, respectively. Twenty-eight isolates had no flagellar antigen, and their H-serotypes were named HNM. Similarity of PFGE patterns between wild-derived isolates and captive-derived isolates was <70%. No plasmid profile was shared between wild-derived and captive-derived isolates. The prevalence of antimicrobial-resistant *E. coli* was 6.5% (n=62) in wild macaques, and these isolates were resistant to cephalothin. We conclude that wild Japanese macaques in Shimokita Peninsula were unlikely to act as a reservoir of pathogenic *E. coli* for humans and that antimicrobial-resistant *E. coli* in wild macaques may be derived from humans.

Key words: Antimicrobial resistance, *Escherichia coli*, genotyping, Japanese macaque, *Macaca fuscata*, zoonosis.

INTRODUCTION

Mammals are sentinels for ecosystem health because they sit at or near the top of food chains (Delahay et al., 2009). Nonhuman primates are well suited for zoonosis research because of their genetic and physiologic similarities to humans (Wolfe et al., 1998). However, zoonotic pathogens are sometimes transmitted from humans to nonhuman primates and can cause population declines in threatened species (Leendertz et al., 2006). Few studies have been conducted on zoonotic pathogens of Japanese macaques (*Macaca fuscata*), the only primate indigenous to Japan.

Shimokita Peninsula, Aomori Prefecture, Japan, has the northernmost distribution of Japanese macaques, the northernmost nonhuman primate, in the world. The Shimokita macaque population is isolated and genetically distinct from other macaque populations in Japan (Kawamoto et al., 2008). This population has been conserved as a national natural treasure and is classified as a locally threatened population by Aomori Prefecture (Aomori Prefecture, 2001). However, the population of macaques in Shimokita increased from 187 in the early 1970s to >1,300 in 2005 (Aomori Prefecture, 2008). Seventeen of 29 macaque troops monitored by the local government caused conflicts with the local people by

damaging crops, intimidating people, and invading homes (Aomori Prefecture, 2008). Such direct or indirect contact between wild macaques and humans might lead to pathogen transmission between them.

Escherichia coli is found naturally as intestinal microflora of many species, including macaques and humans. Most *E. coli* strains are commensal, but some are pathogenic to humans. In particular, Shiga toxin-producing *E. coli* (STEC), such as serotype O157:H7, is one of the most important human pathogens in industrialized countries (Beutin, 2006). Although cattle are considered to be the most important reservoir of STEC (Caprioli et al., 2005), wildlife such as deer are associated with STEC (Asakura et al., 1998).

The occurrence of antimicrobial resistance among pathogenic and commensal bacteria is a significant problem affecting medical treatment of infectious diseases. Humans (Ishikawa et al., 2005), food animals (Asai et al., 2005), and wildlife (Gilliver et al., 1999) may act as reservoirs of drug-resistant bacteria through the food chain. Antimicrobial resistance was found to be widespread in enterobacteria from wild rodents in England (Gilliver et al., 1999). However, in Finland, where antimicrobial use is much less common than in England, antimicrobial resistance was found to be almost absent in enterobacteria from wild ungulates and voles (Österblad et al., 2001). The widespread occurrence of antimicrobial resistance in bacteria in wildlife populations may be caused by environmental pollution of antimicrobials or resistant bacteria through human activities such as antimicrobial use in medicine and agriculture.

Our goals for this study were to 1) investigate the risk of transmission of pathogenic *E. coli*, especially STEC, from wild macaques to humans; 2) investigate the transmission of *E. coli* within the wild macaque population; and 3) determine the prevalence of antimicrobial-resistant *E. coli* and estimate the extent of human impact on the wild macaque population.

MATERIALS AND METHODS

Study areas, macaque behavior, and fecal sample collection

Fecal samples were collected in northwestern (41°25′N, 140°50′E) and southwestern (41°8′N, 140°49′E) Shimokita Peninsula, Aomori Prefecture, Japan (Fig. 1), in December 2005 and from October to December 2006. Thirteen troops were surveyed: 12 troops of wild Japanese macaques and a captive troop kept at Wakinosawa Monkey Park (Table 1). Troops that visit farmland seasonally or throughout the year and forage crops were classified as "high damage troops," and those that visit farmland sometimes or seasonally and forage grass plants, grains, or vegetables left around fields were classified as "low damage troops." Habituation levels of the troops were further classified as high (troops that do not run away from humans and pass by humans at close range), intermediate (troops that do not run away but keep away from humans) and low (troops that run away from humans). The local government attached radiotelemetry collars to one or two macaques in each group. Solitary males that did not belong to any troop also were surveyed. Fresh feces (≤1 day old) on the ground were collected during the tracking of troops. Macaque troops were identified from their telemetry information or the location in which they were observed. Fecal samples were stored in sterilized bags at 4 or −20 C until isolation attempts.

Isolation and identification of *E. coli*

One gram of each fecal sample was incubated twice in modified *E. coli* broth (Oxoid, Basingstoke, Hampshire, UK) with novobiocin (20 μg/ml) for 24 hr at 42 C for the recovery of freeze-injured *E. coli* cells (Hara-Kudo et al., 2000). The enrichment culture was selected by the immunomagnetic separation (IMS) method with Dynabeads anti-*E. coli* O157 (Invitrogen, Carlsbad, California, USA). After IMS, the culture was incubated 24 hr at 37 C on CHROMagar O157 TAM plates (CHROMagar, Paris, France) and Tricorol plates (EL-MEX, Tokyo, Japan). Up to four suspected colonies per sample were selected and identified by biochemical tests with triple sugar iron medium (Eiken, Tokyo, Japan); lysine, indole, motility medium (Eiken); VP semisolid medium (Eiken); and Simmon's citrate agar (Eiken). Confirmed *E. coli* isolates were serotyped by agglutination tests with *E. coli* antisera "Seiken" (Denka-Seiken, Tokyo, Japan) following the manufacturer's instructions.

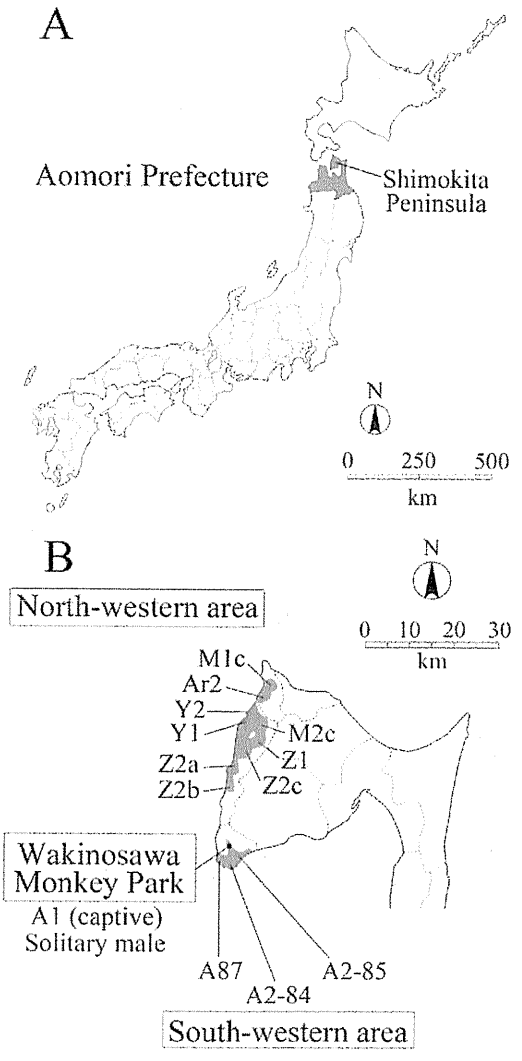

FIGURE 1. Study site and the locations of Japanese macaque (*Macaca fuscata*) troops studied for antibiotic resistant *Escherichia coli* strains. (A) Location of Aomori Prefecture (shaded) and Shimokita Peninsula. (B) Total habitat (shaded) of wild macaque troops surveyed in this study (Aomori Prefecture, 2004; Matsubara, 2006), showing Wakinosawa Monkey Park and approximate locations of each surveyed troop. Habitats of neighboring troops overlapped in marginal regions. Fecal samples of solitary males were collected in Wakinosawa Monkey Park.

Genotyping of *E. coli*

PCR for the virulence genes for intimin (*eae*), Shiga toxin (*stx*), LT (*elt*), and ST (*est*) was performed on isolates for which serotypes had been determined. DNA extracted with a DNeasy™ Tissue Kit (QIAGEN, Hilden, Germany), or boiled enrichment culture was used as templates. Oligonucleotide primers (Table 2) for PCR were as follows: SK1, SK2 for *eae* (Oswald et al., 2000), Vtcom-u, Vtcom-d for *stx* (Yamasaki et al., 1996), LT_L, LT_R for *elt* (Toma et al., 2003), and AL65, AL125c for *est* (Toma et al., 2003). PCR was performed under the following conditions: initial denaturation for 5 min at 94 C, 30 cycles of 1 min at 94 C, 1 min at 55 C, 1 min at 72 C, and a final extension step of 7 min at 72 C. PCR amplicons were electrophoresed on 2% SeaKem GTG agarose gel (Takara, Otsu, Shiga, Japan) in 1× TAE buffer.

TABLE 1. Human-macaque (*Macaca fuscata*) interactions in Aomori Prefecture, Japan (2005–2006).

Area[a]	Troop	Damage level[b]	Habituation level[b]
NW	M1c[c]	H	L
	Ar2[c]	H	I
	Y1[c]	H	I
	Y2[c]	H	I
	M2c[c]	L	L
	Z1	L	L
	Z2a[c]	H	I
	Z2b[c]	H	I
	Z2c	H	L
SW	A2-84[c]	H	H
	A2-85[c]	H	H
	A87[c]	L	H

[a] NW = northwest; SW = southwest.

[b] H = high; L = low; I = intermediate.

[c] One or two individuals in the troop carried radiotelemetry transmitters.

Plasmids were extracted using the methods of Kado and Liu (1981). Extracted plasmid DNA was electrophoresed on 0.8% PFC agarose gel (Bio-Rad Laboratories, Hercules, California, USA) in 1× TBE buffer with a Subcell 192 system (Bio-Rad Laboratories) for 120 min at 100 V.

Pulsed-field gel electrophoresis (PFGE) was performed on dominant isolates. *Escherichia coli* cells in L-broth were suspended in 150 µl of 1× TE buffer (10 mM Tris [pH 8.0] and 1 mM EDTA [pH 8.0]). They were mixed with 150 µl of 1.2% Certified Megabase agarose (Bio-Rad Laboratories) and solidified with a 0.7-mm plug mold (Bio-Rad Laboratories). Plugs were incubated in 2 ml of lysis buffer (0.5 M EDTA [pH 8.0] and lysozyme [3 mg/ml]) for 6–18 hr at 37 C. After lysis, plugs were incubated in 1 ml of proteinase K buffer (0.5 mM EDTA [pH 8.0], proteinase K [1 mg/ml], and 1% [wt/vol] *N*-lauroylsarcosine) for

TABLE 2. Sequences of primers used to detect the virulence genes for *Escherichia coli* from macaques (*Macaca fuscata*) in Aomori Prefecture, Japan (2005–2006).

Primer	Sequence	Target gene	Size (bp)
SK1	5′-CCCGAATTCGGCACAAGCATAAGC-3′	*eae*	881
SK2	5′-CCCGGATCCGTCTCGCCAGTATTCG-3′		
VTcom-u	5′-GAGCGAAATAATTTATATGTG-3′	*stx*	518
VTcom-d	5′-TGATGATGGCAATTCAGTAT-3′		
LT_L	5′-TCTCTATGTGCATACGGAGC-3′	*elt*	322
LT_R	5′-CCATACTGATTGCCGCAAT-3′		
AL65	5′-TTAATAGCACCCGGTACAAGCAGG-3′	*est*	147
AL125c[a]	5′-TTAATAGCACCCGGTACAAGCAGG-3′		

[a] We altered the sequences of the primer from that of AL125 reported by Toma et al. (2003).

12–24 hr at 55 C. Proteinase K was inactivated with 1 ml of Pefabloc solution (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0], and 2 mM Pefabloc) for 30 min at room temperature. After washing and equilibration, DNA digestion was performed with XbaI (25 U per sample). PFGE was performed on 1% PFC agarose gel (Bio-Rad Laboratories) in 0.5× TBE buffer containing 100 μM thiourea (Liesegang and Tschape, 2002) with CHEF-DR II (Bio-Rad Laboratories). The electrophoretic profile was converted into a TIFF file with Foto/Eclips™ (Fotodyne, Hartland, Wisconsin, USA) and stored in the database with BioNumerics® version 3.0 (AppliedMath, Sint-Martens Latem, Belgium). The similarity among isolates was compared with the unweighted pair-group method with arithmetic mean with a tolerance of 1.5%. The *E. coli* isolates were identified from their PFGE profiles according to Tenover et al. (1995).

Screening for antimicrobial resistance of *E. coli*

Disc diffusion and agar dilution methods (Japanese Society of Antimicrobials for Animals, 2004) were performed to determine microbial sensitivity according to Clinical and Laboratory Standards Institute (CLSI, Wayne, Pennsylvania, USA). Disc diffusion method was performed with BD Sensi-Disc (BD Biosciences, Franklin Lakes, New Jersey, USA). Tested antimicrobials were ampicillin (ABPC, 10 μg), penicillin G (10 units), kanamycin (30 μg), gentamycin (10 μg), streptomycin (SM, 10 μg), erythromycin (15 μg), tetracycline (30 μg), chloramphenicol (30 μg), colistin (10 μg), fosfomycin (50 μg), vancomycin (30 μg), cefazolin (30 μg), cephalothin (CET, 30 μg), cefmetazole (30 μg), cefotiam (30 μg), cefoperazon (75 μg), latamoxef sodium (30 μg), cefotaxime (30 μg), nalidixic acid (30 μg), norfloxacin (10 μg), ofloxacin (5 μg), ciprofloxacin (5 μg), sulfamethoxazole-trimethoprim (23.75/1.25 μg), and sulfamethizole (250 μg). Each isolate was classified as susceptible, intermediate, or resistant, depending on the growth inhibition diameter. Minimum inhibitory concentration of suspected resistant isolates were determined by the agar dilution method with Mueller-Hinton agar (Nissui, Tokyo, Japan) according to the recommendations of CLSI. In brief, suspected resistant isolates were cultured in 1 ml of Mueller-Hinton broth (Difco, Detroit, Michigan, USA) for 2–6 hr at 35 C to match the turbidity of a 0.5 McFarland standard. A 10-fold dilution of cultures was inoculated into Mueller-Hinton agars (Nissui) containing antimicrobials with a microplanter. Resistance to each antimicrobial was determined according to the interpretative breakpoints defined by CLSI (Table 3).

Statistical analysis

The prevalence of antimicrobial resistant *E. coli* isolates from wild macaques was compared with that of captive macaques by Fisher's exact test. Differences were considered significant at $P<0.05$. Statistical analysis was performed using Excel 2007 (Microsoft, Redmond, Washington, USA) with the add-in software Statcel 2 (Yanai, 2004).

RESULTS

Escherichia coli isolates

Escherichia coli was detected in 159 of 265 fecal samples (60%) from wild macaques and in 17 of 20 samples (85%) from captive macaques. In total, 290 strains (248 from wild macaques and 42 from captive macaques) were isolated (Table 4). Six serotypes were identified in the isolates in 2005 and 33 strains of

TABLE 3. Concentrations of antimicrobials and interpretative breakpoints for antimicrobial resistance testing of *Escherichia coli* strains recovered from macaques (*Macaca fuscata*) in Aomori Prefecture, Japan (2005–2006).

Antimicrobial[a]	Concentration[b] (μg/ml)	MIC[c] breakpoint (μg/ml)		
		S	I	R
ABPC	0.0625–512	≤8	16	≥32
CET	0.0625–512	≤8	16	≥32
SM	0.0625–512	≤8	16	≥32

[a] ABPC = ampicillin; CET = cephalothin; SM = streptomycin.

[b] In Mueller-Hinton agar.

[c] MIC = minimum inhibitory concentration.

four serotypes (O8:H34, O8:H42, O8:HUT, and O157:H42) were genotyped by PFGE and plasmid profiles. O- and H-serotypes of the 26 isolates were not typed by the above-mentioned typing antisera and were named OUT and HUT, respectively. Nineteen serotypes were identified in isolates from 2006 and 75 strains of six serotypes (O8:H6, O8:HUT, O103:H27, O103:HNM, OUT:H27, and O119:H21) were genotyped by PFGE and plasmid profiles. Twenty-eight isolates had no flagellar antigen, and their H-serotypes were named HNM. None of the virulence genes examined (*eae*, *est*, *elt*, and *stx*) were detected in the isolates. The isolates were classified into 15 clusters based on the PFGE patterns (Fig. 2). The isolates in each cluster had at least 80% similarity. Seventeen plasmid profiles were observed and named as types a–q (Table 5).

Distribution of serotypes and genetic similarity

Serotype O8 strains were isolated from numerous wild macaque troops in 2005 and 2006 but not from any captive macaques (Fig. 3). Serotype O8 strains consisted of eight clusters (clusters 3 and 8–14; Fig. 2). All isolates of serotype O103:H27 were classified into cluster 2 (Fig. 2), and seven of 12 isolates had identical plasmid profiles (Fig. 2).

The relatedness between *E. coli* isolates from wild macaques and captive macaques was low by serotyping, PFGE, and plasmid profiling. Serotype O157:H42 and O119:H21 strains were isolated from captive macaques in 2005 and 2006, respectively (Fig. 3). Serotype O157:H42 strains had four unique PFGE patterns, and their similarity to wild-derived isolates was <50% (Fig. 2). Serotype O119:H21 strains had a single PFGE pattern, and their similarity to wild-derived isolates was <70% (Fig. 2). No plasmid profile was shared between wild- and captive-derived isolates (Fig. 2).

Antimicrobial resistance

Microbial sensitivity tests were conducted on 33 isolates from 27 fecal samples in 2005 and on 50 isolates from 44 fecal

TABLE 4. Numbers of fecal samples collected and numbers of *Escherichia coli* isolates obtained from Japanese macaques (*Macaca fuscata*) in Aomori Prefecture, Japan (2005–2006).

Area	No. fecal samples			No. *E. coli* isolates		
	2005	2006	Total	2005	2006	Total
Northwestern	56	98	154	57	93	150
Southwestern	35	76	111	12	86	98
Captive	9	11	20	32	10	42
Total	100	185	285	101	189	290

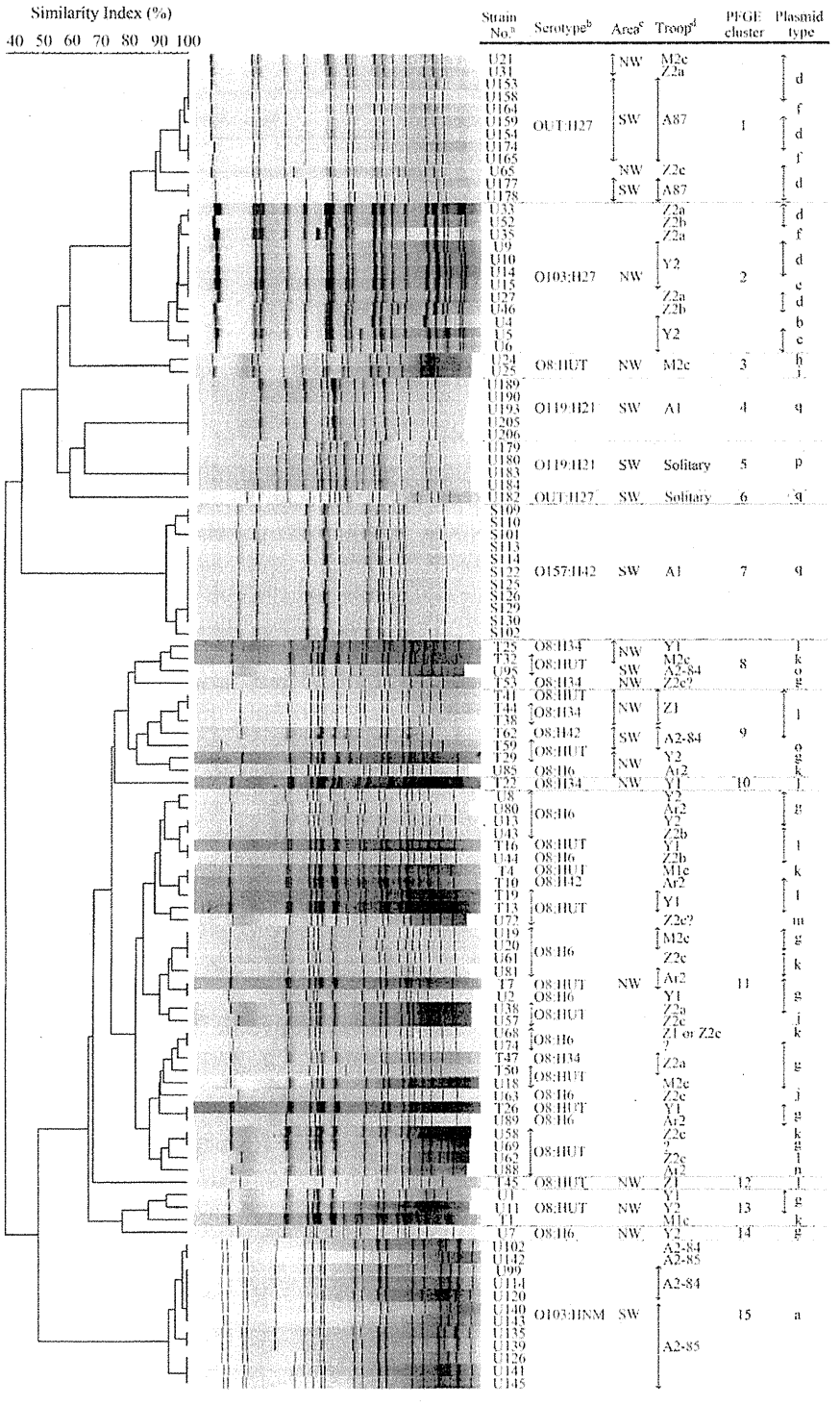

FIGURE 2. Dendrogram of pulsed-field gel electrophoresis patterns of *Escherichia coli* isolates from Japanese macaques (*Macaca fuscata*) sampled in Shimokita Peninsula Aomori Prefecture, Japan (2005–2006). Similarity index scale at top left. (a) Strains beginning with S or T isolated in 2005; strains beginning with U isolated in 2006. (b) UT = untypable; NM = nonmotile. (c) NW = northwest; SW = southwest. (d) Solitary = solitary males not belonging to any troop; Z2c? = likely to be Z2c troop; Z1 or Z2c = troop may have been Z1 or Z2c; ? = unknown troop.

TABLE 5. Plasmid types from *Escherichia coli* isolates from Japanese macaques (*Macaca fuscata*) in Shimokita Peninsula, Aomori Prefecture, Japan (2005–2006).

Plasmid type	Size[a] (kbp)
a	186
b	143, 88, 52
c	173, 52
d	143, 68, 52
e	143, 68
f	143, 88, 68, 52
g	101
h	143, 75, 53
i	143, 75
j	53
k	101, 44
l	101, 53
m	101, 53, 44
n	101, 75, 44
o	101, 75, 53
p	5.3, 3
q	No plasmid

[a] Plasmid sizes were estimated from the migration distances of each band.

samples in 2006. Fifty-eight isolates were resistant to antimicrobials by disc diffusion (17 to CET, 13 to ABPC, 27 to CET and ABPC and one to SM). Minimum inhibitory concentrations to ABPC, CET and SM of these 58 isolates were determined by agar dilution (Japanese Society of Antimicrobials for Animals, 2004). The concentration of each antimicrobial in agar was 0.0625–512 μg/ml. Four isolates (U20, U38, U44, and U61) were resistant to CET. These four isolates came from four troops from the northwestern site in 2006. The prevalence of resistant *E. coli* was 6.5% in wild macaques ($n=62$) and 0% in captive animals ($n=9$), but the difference was not significant (Fisher's exact test, $P=0.57$; $n=71$).

DISCUSSION

We isolated no pathogenic *E. coli* strains from wild Japanese macaques. Wild macaques are unlikely to act as reservoirs of pathogenic *E. coli*. However, genomic similarity has been observed between enteropathogenic *E. coli* (EPEC) from humans and monkeys, and monkeys were suspected as a reservoir of EPEC in Brazil (Carvalho et al., 2007). Therefore, it is important to continue monitoring for pathogenic *E. coli* among wild macaques, especially where they coexist with humans.

Phenotyping and genotyping revealed that wild macaques of more than one troop carried genetically similar *E. coli* strains. Some troops with similar isolates were in adjacent areas, but some were in separate areas. It is possible that the common strains among wild macaques in our surveyed area are specifically adapted to the internal environment of these animals. The fecal microflora of wild Japanese macaques in snowy areas was different from that of captive macaques, possibly because of the bark-eating habits of the wild macaques (Benno et al., 1987). In our study site, wild macaques mainly fed on dormant buds and bark in winter (Nakayama et al., 1999). Serotype O8, the most frequent serotype in wild macaques, was not isolated from captive macaques, suggesting that serotype O8 strains may be adapted to the internal environment of bark-eating wild macaques.

Another possibility for the similarity of *E. coli* isolates is the transmission of *E. coli*. Some strains, such as serotypes O103:H27, O103:HNM, and OUT:H27, were isolated from wild macaques of particular troops. Those isolates had high genetic similarities in PFGE and plasmid profiles (Fig. 2). Most macaque troops carrying those isolates lived in adjacent areas, suggesting that genetically close strains may be spread in this subset of the Shimokita macaque population. Perhaps other wild animals spread these isolates or the isolates spread environmentally. To examine *E. coli* transmission in the wild macaque population, comprehensive analysis of *E. coli* not only from wild macaques but also from other wild animals and soil in the same area is needed.

Antimicrobial-resistant bacteria among wild animals are thought to be derived from humans or domestic animals through

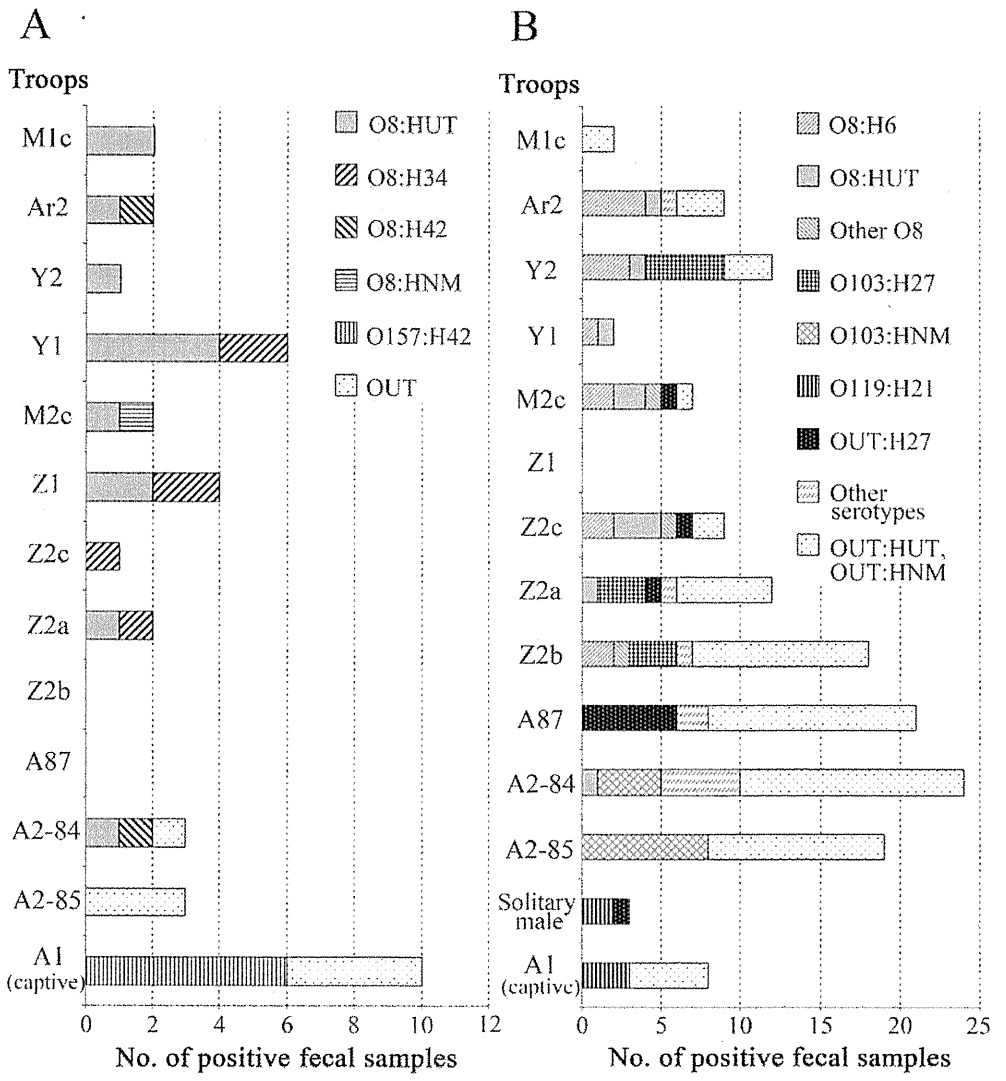

FIGURE 3. Serotypes of *Escherichia coli* isolates from 13 troops and from solitary males of Japanese macaques (*Macaca fuscata*) sampled in Aomori Prefecture, Japan in (A) 2005 and (B) 2006. Serotypes of isolates from unidentified troops not shown. UT = untypable; NM = nonmotile.

human activity, such as agriculture (Österblad et al., 2001). Blanco et al. (2009) reported that antimicrobial resistance in wild birds was associated with agricultural manure. In Japan, wild animals in densely populated areas carried antimicrobial-resistant bacteria more often than those in sparsely populated areas (Ogawa et al., unpubl.). In our study, the prevalence of antimicrobial-resistant *E. coli* isolates was very low among both wild and captive macaques in Shimokita Peninsula. Both wild and captive macaques had direct or indirect contact with humans through their foraging in agricultural land or from captivity. Nevertheless, the low prevalence of resistant *E. coli* in the macaque population suggests that contact with humans did not affect the spread of resistant *E. coli* in macaques.

Humans and domestic animals are possible sources of resistant *E. coli* strains found in wildlife. The patterns of antimicrobial resistance are different between

humans and domestic animals. Most of the resistant *E. coli* isolates from food animals were found to be sulfadimethoxine-resistant, oxytetracycline-resistant, and dihydrostreptomycin-resistant (Kijima-Tanaka et al., 2003). Cephem-resistant bacteria were rarely detected in Japanese livestock (Asai et al., 2005), but they have been found in companion animals (Pedersen et al., 2007) and humans (Ishikawa et al., 2005). In this study, all four resistant *E. coli* isolates from wild macaques were cephalothin-resistant. They were not derived from livestock and the likelihood that they were derived from companion animals is low, because few veterinary clinics are located in our study area (Ministry of Agriculture, Forestry and Fisheries, 2009). Therefore, resistant *E. coli* isolates in macaques possibly were derived from humans. However, macaque isolates were not compared with those of humans or domestic animals in this study. Further study is needed to determine the origin of the resistant *E. coli* strains from wild macaques.

Our results indicate that wild Japanese macaques in Shimokita Peninsula are probably not reservoirs of pathogenic *E. coli*, and they also suggest that bacterial transmission from humans or domestic animals to wild macaques is rare. However, the possibility remains that antimicrobial-resistant *E. coli* isolates from wild macaques were derived from humans. Given that the contact between wild macaques and humans is increasing, epidemiologic studies of pathogenic and resistant strains should be continued for the conservation of wild macaques as well as for public health.

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コクシエラ・クラミジア感染症

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要　旨

偏性細胞内寄生体を原因とするQ熱およびオウム病について記述した．Q熱はコクシエラ菌による疾患である．ヒトは病原体を含む粉塵の吸入により感染し，呼吸器疾患や心内膜炎などを呈する．我が国のQ熱は感染源や伝播経路がはっきりしていない．オウム病はオウム病クラミジアを病原体とし，愛玩用鳥類やドバトからヒトに感染し，肺炎などの呼吸器感染症を引き起こす疾患である．非定型肺炎の1つとして的確な治療が必要である．

コクシエラ感染症

コクシエラ感染症は偏性細胞内寄生性細菌である *Coxiella burnetii* を起因菌とする人獣共通感染症である[1]．ヒトにおけるコクシエラ感染症はQ熱（Q fever）と呼ばれる．宿主は哺乳動物，鳥類およびダニを主とする節足動物である．ヒトは病原体を含む粉塵を環境から吸入することによって感染する．急性症状はインフルエンザ類似の呼吸器症状であり，慢性に移行すると心内膜炎を起こす．我が国では感染症法において第四類に指定され，診断した医師は直ちに届け出る義務がある．しかしながら，我が国におけるQ熱は感染源や伝播経路がはっきりせず，診断法についてもいまだに過渡的な状況にある感染症である．

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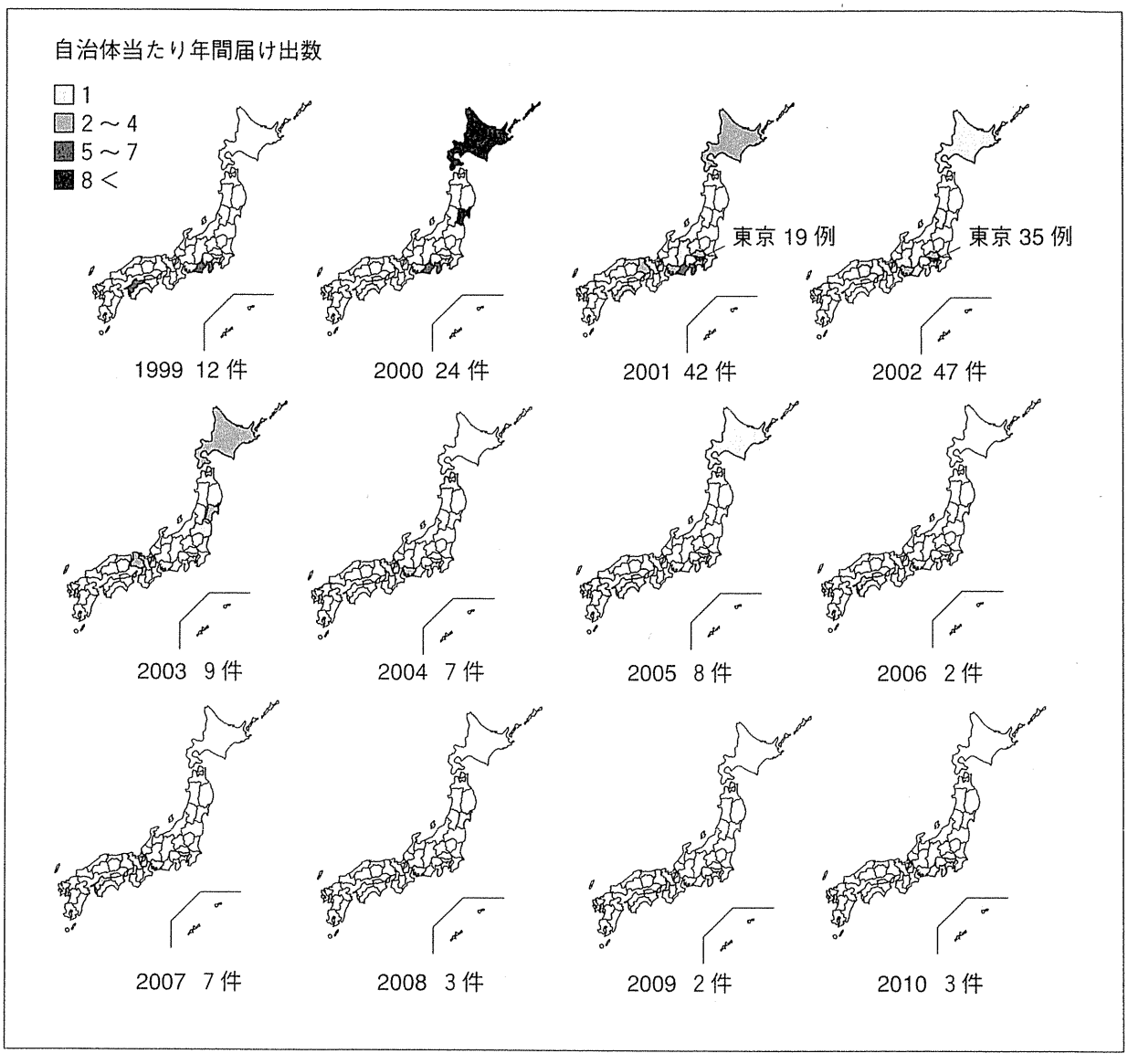
1．病原体

C. burnetii はレジオネラ目に分類され，コクシエラ科コクシエラ属に属する偏性細胞内寄生性小桿菌である．生物学的にリケッチアとの関連性はない．大きさは 0.2～0.4×1.0 μm で，多形性を示す．*C. burnetii* は細胞質の空胞内に見られる．世代時間は 20～45 時間である．最近，無生物培地における培養が可能であるという報告がなされた[2]．この無生物培地を用いた動物組織からの *C. burnetii* の分離ならびに形質転換も報告された[3]．

2．疫　学

Q熱は世界的に広く分布しており[4]，1999年から 2004 年において 12ヵ国から 18 件の集団発生報告があり，各発生当たりの患者数は 2 人から 289 人であった．感染源はヒツ

図1　都道府県別のQ熱届け出数の年次推移（1999年4月から2010年）

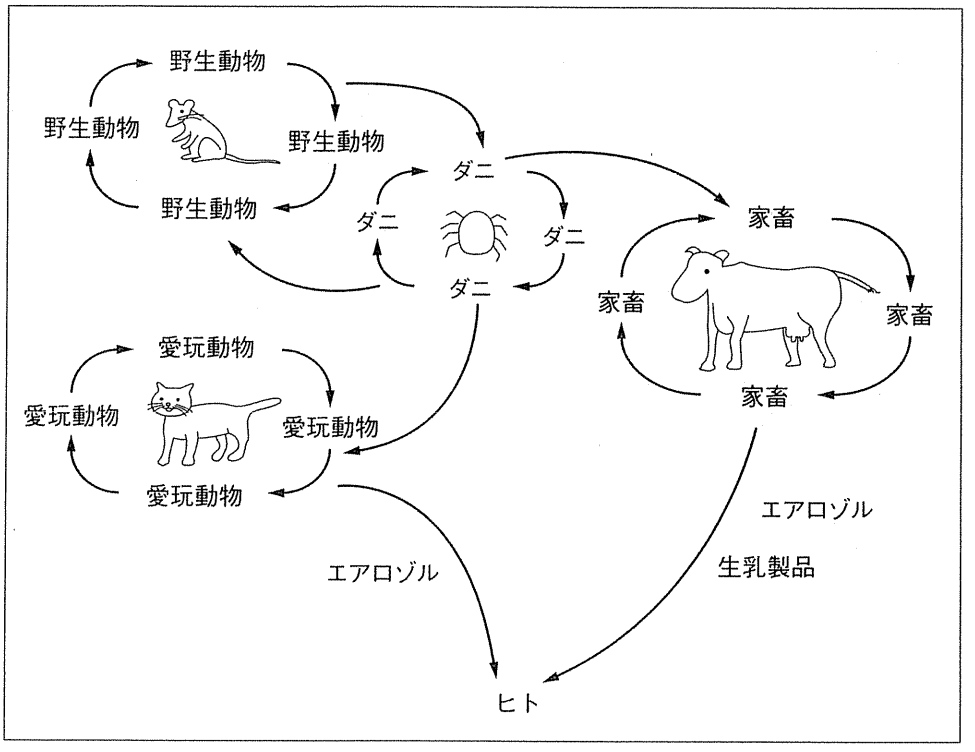


ジ，ヤギ，野生動物，イヌ，ネコであった．また，感染源が不明な集団発生も2件あった．オランダでは2007年以降Q熱患者が急増し，問題となっている[5]．2007年から2010年の4年間で4,000症例が報告された．2007年以前は年間20症例程度であったという．この急増の原因は明らかではないが，都市近郊の農場で飼育されているヤギやヒツジにおけるコクシエラ感染症との関連性が示唆されている．

日本におけるコクシエラ感染症は，1999年以降届け出が義務づけられている．1999年から2002年まで届け出数は急増した（厚生労働省/国立感染症研究所 感染症週報，図1）．しかし，2003年以降は10例前後の届け出となった．2008年から2010年は2～3件の報告数となっている．都道府県別では，2001年と2002年における東京都の届け出数がそれぞれ19件および35件と多かった．諸外国では市中肺炎の原因として *C. burnetii* も診断の対象とされているが，日本ではあまり考慮されていないことが危惧されている[6]．

C. burnetii は，自然界においてダニと哺乳動物を宿主に感染環が成立している[7]（図2）．40種以上のダニから *C. burnetii* が分離されているが，家畜動物やヒトにおける感

図2　コクシエラ菌の自然界における感染環



染環の維持にダニは重要ではない．*C. burnetii* はダニ体内で増殖し，ダニの糞中に大量に含まれるようになる．このダニの糞が環境を汚染し，感染源となる．ヨーロッパにおいて重要な感染源は，ウシ，ヒツジおよびヤギであると考えられている．これらの動物が *C. burnetii* に感染すると，子宮や乳腺に局在する．ネコ，ウサギ，イヌも都市部では感染源になりうる．

日本における動物のコクシエラ症に関する調査では，健康牛の 16.9～49.6% に抗体陽性が認められ，繁殖障害牛では 60～84% に抗体が認められた[8]．*C. burnetii* 分離においても，ウシ，イヌ，ネコおよびダニからの分離報告がなされている．

3．感染経路

ヒトにおける伝播経路はエアロゾルの吸入および経口経路が主である．感染動物の体液，体毛などに由来するエアロゾルを吸引することによって感染する．また，汚染された動物の乳の飲用による感染も知られている．ヒトからヒトへの伝播はまれである．最近，性行為による感染が報告された[9]．

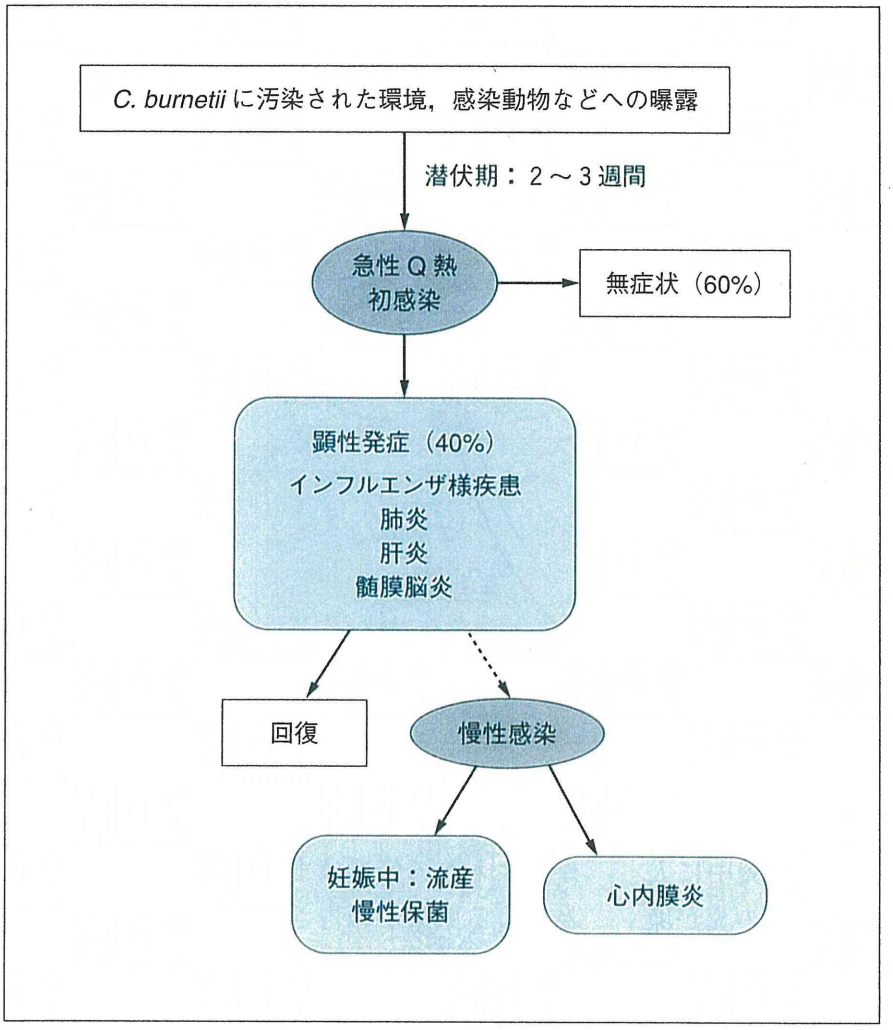
4．ヒトのQ熱の臨床

ヒトのQ熱の症状はインフルエンザ様の急性熱性疾患を主徴とし，ウイルスおよび細菌による呼吸器疾患と誤診されやすく，鑑別が難しい．したがって，病原学的または血清学的診断によらなければならない．臨床像は急性と慢性に分けられる（図3）．急性例の潜伏期は 14～26 日で，感染量が多いと短い．一般に高齢者が発症しやすいと言われている．予後は一般に良く，多くは約 12 日で下熱し回復する．治療が遅れると死の転帰をとることがある．慢性の場合は，急性感染後の回復期から心内膜炎へと移行する．

5．病原学的および血清学的診断

コクシエラ菌は，バイオセーフティレベル3の病原体として指定されている．病原体分

図3　Q熱の病態発生



離は十分な注意が必要である．コクシエラ症の病原学的診断は実験小動物，発育鶏卵および培養細胞接種法のいずれかの方法によって行われる．最近，人工培地でコクシエラ菌を培養できることが報告された[2)3)]．今後の発展が望まれる．

コクシエラ症の血清学的診断には，補体結合反応（CF），間接蛍光抗体法（IFA），酵素抗体法（ELISA）などがある．IFA およびELISA が現在のところ最も一般的に用いられている．ELISA 抗体価は IFA 抗体価とよく相関する．いずれの方法においても単独検体による診断はできない．ペア血清を用い，抗体価の上昇が確認された場合にのみ，確定診断に用いることができる．

6．コクシエラ感染症のまとめ

コクシエラ感染症は，欧米において市中肺炎および人獣共通感染症としてよく知られている．一方，日本におけるコクシエラ感染症は，血清学的に広く存在することが示唆されているにもかかわらず，実際の症例は年間数例程度である．また，家畜衛生における意義も不明である．しかしながら，近年ヨーロッパの一部の国では発生数が増加していることから，日本においても注意が必要な人獣共通感染症である．

クラミジア感染症

クラミジア感染症における人獣共通感染症には，オウム病（*Chlamydia psittaci* 感染症），