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Amino Acid Substitutions in the S2 Region Enhance Severe Acute Respiratory Syndrome Coronavirus Infectivity in Rat Angiotensin-Converting Enzyme 2-Expressing Cells▽

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To clarify the molecular basis of severe acute respiratory syndrome coronavirus (SARS-CoV) adaptation to different host species, we serially passaged SARS-CoV in rat angiotensin-converting enzyme 2 (ACE2)-expressing cells. After 15 passages, the virus (Rat-P15) came to replicate effectively in rat ACE2-expressing cells. Two amino acid substitutions in the S2 region were found on the Rat-P15 S gene. Analyses of the infectivity of the pseudotype-bearing S protein indicated that the two substitutions in the S2 region, especially the S950F substitution, were responsible for efficient infection. Therefore, virus adaptation to different host species can be induced by amino acid substitutions in the S2 region.

The 2002 to 2003 epidemic of severe acute respiratory syndrome (SARS) was caused by SARS coronavirus (SARS-CoV) infection. Initially, SARS-CoV was thought to be transmitted first from marketplace animals to humans and then by human-to-human spread (3, 7, 8). Although marketplace animals may be the immediate source of the SARS-CoV found in humans, SARS-CoV has been detected in other wild animals, e.g., civet cats, raccoon dogs (7), ferrets, and cats (15), suggesting that SARS-CoV may have a broad host range. In addition, rats are suggested to have been an animal vector in the SARS outbreak in the Amoy Gardens in Hong Kong (20). Recently, horseshoe bats have been reported to be a natural reservoir of coronaviruses close to SARS-CoV, and it is suggested that bats are candidate natural reservoirs of SARS-CoV (9, 13). In order to understand how the virus jumped to humans, it is important to elucidate the molecular mechanism of SARS-CoV adaptation to different species.

The SARS-CoV S protein mediates virus entry into cells expressing the receptor molecule angiotensin-converting enzyme 2 (ACE2) (12). The receptor binding domain (RBD), located on the S1 region, is believed to be the critical determinant of virus-receptor interaction (10, 27). It has been shown that amino acid substitutions on the RBD are associated with the SARS-CoV from palm civets adapted to humans (14). Furthermore, a single amino acid substitution on the RBD caused by serial in vivo passage of SARS-CoV in rats was strongly associated with increased infection of rat ACE2-expressing cells (18). Thus, substitution(s) of amino acid residues on the RBD may be one of the critical molecular determinants of SARS-CoV adaptation. On the other hand, in the case of mouse hepatitis virus, one or more amino acid substitutions of the S2 region, in combination with those of the S1 region, are intimately involved in receptor binding and extended host range (2, 16, 19). The presence of a neutralizing epitope within the S2 region of the SARS-CoV suggests that the S protein binding to the host cell surface not only relies on the S1 region but also depends on the global protein structure, including the S2 region (4, 28, 29). These studies suggested that several factors, including amino acid substitutions in the S2 region, in addition to those in the RBD in the S1 region, play roles in determining SARS-CoV infectivity. Thus, analyses of the entire amino acid sequence of the S protein may be necessary to understand the molecular mechanisms of viral adaptation to different species.

Notably, SARS-CoV S protein-mediated entry into cells expressing rat ACE2 has been shown to be extremely low (11, 14). Since the coronavirus serially passaged in vitro acquires amino acid substitutions that might be relevant to virus adaptation to different types of cells (5, 23), we speculated that in vitro passage of SARS-CoV in cells expressing species-specific ACE2 would induce viral adaptation to different animal species. To examine SARS-CoV adaptation to rat ACE2, we compared replication efficiencies for SARS-CoV serially passaged in rat ACE2-expressing cells and for a parental SARS-CoV strain when inoculated to cells expressing rat ACE2.

The SARS-CoV Frankfurt-1 strain was inoculated at a multiplicity of infection (MOI) of 0.01 PFU/cell onto Chinese hamster ovary (CHO) cells transiently transfected with the expression plasmid pcDNArat ACE2, which encodes rat ACE2 (18). The expression of ACE2 proteins was confirmed by Western blotting (Fig. 1A). The plasmids were transfected into the CHO cells at efficiencies of about 80%, as estimated by indirect immunofluorescence methods (data not shown). The virus, which was obtained from the culture supernatants of Frankfurt-1-infected rat ACE2-expressing CHO cells, was reinoculated at an MOI of 0.01 PFU/cell onto fresh CHO cells expressing rat ACE2. The culture supernatants were collected

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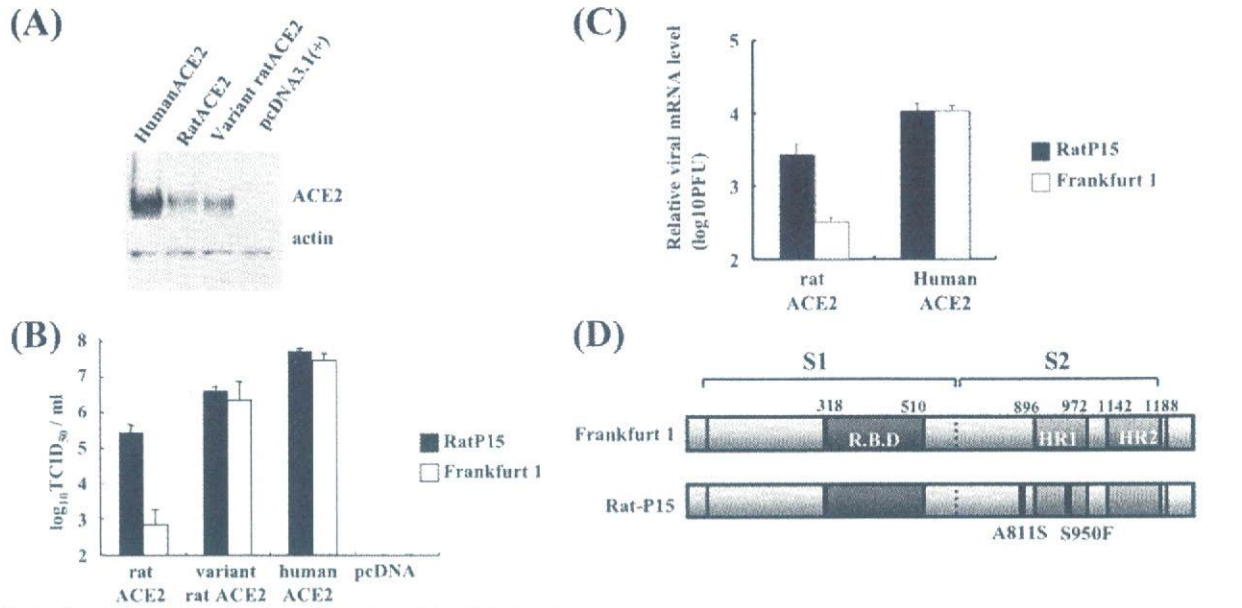

FIG. 1. Comparison of replication efficiencies of the SARS-CoV Rat-P15 strain serially passaged in rat ACE2-expressing cells and that of a parental SARS-CoV strain (Frankfurt-1). (A) Expression of ACE2 proteins. CHO cells were transfected with plasmid pTargeT-hACE2 (6), pcDNArat ACE2, and pcDNArat ACE2MUT2 (18), which encode human ACE2, rat ACE2, and variant rat ACE2 with amino acid residues 82 to 84 (NYS) corresponding to human ACE2 (MYP), respectively. Equal volumes of cell lysates were analyzed by Western blotting using a goat antibody specific for human ACE2 (6) or β-actin. The low signal intensity on rat ACE2 is due to the lower reactivity of the antiserum to rat ACE2. (B) Replication of viruses in CHO cells expressing rat ACE2, variant rat ACE2, or human ACE2. Rat-P15 or a parent Frankfurt-1 strain was inoculated to CHO cells transfected with expression plasmids pcDNArat ACE2, pcDNArat ACE2MUT2, pTargeT-hACE2, or the pcDNA3.1(+) vector. After 72 h, the replications of the virus in the cells were determined as 50% tissue culture infectious doses ($TCID_{50}$)/ml on Vero E6 cells. (C) Assessment of virus entry of Rat-P15 on rat ACE2-expressing cells. CHO cells were transfected with expression plasmids encoding rat ACE2 or human ACE2. After 48 h, 10,000 PFU of Rat-P15 or a parent Frankfurt-1 strain was inoculated. After 5 h, the culture medium was removed, and viral RNAs were isolated from the infected cells. Virus entry efficiency was estimated by quantification of the mRNA9 level with a real-time PCR assay (17). (D) Schematic representation of S proteins of Rat-P15 and Frankfurt 1. Amino acid substitutions at residues 811 and 950 in the S2 region of Rat-P15 are shown. The locations of the RBD, heptad repeat 1 (HR1), and HR2 are shown as filled boxes.

after 48 hours postinfection (hpi). All the following passages were performed by inoculation of the virus at an MOI of 0.002 PFU/cell and by collection of the culture supernatants at 72 hpi. Passages were performed 15 times, and the virus (Rat-P15) was obtained from culture supernatants. In order to examine whether serial passages of SARS-CoV makes the virus replicate efficiently in rat ACE2-expressing cells, Rat-P15 or a parent Frankfurt-1 strain was inoculated onto CHO cells that express rat ACE2. After 72 h, the virus titers of the culture supernatants were determined. The replication of Rat-P15 was higher than that of the parent Frankfurt-1 strain in rat ACE2-expressing CHO cells (Fig. 1B). In contrast, the replication of Rat-P15 was similar to that of parent Frankfurt-1 strain in CHO cells expressing human ACE2 or variant rat ACE2 with amino acid residues 82 to 84 (NYS) corresponding to human ACE2 (MYP) (18) (Fig. 1B). These results indicate that Rat-P15 came to replicate more efficiently in rat ACE2-expressing cells.

To examine whether the Rat-P15 strain acquired the ability to infect rat ACE2-expressing cells more efficiently than the parent Frankfurt-1 strain, viral infection was determined by quantitative real-time PCR assay after a shorter incubation period (17). Viruses were inoculated onto CHO cells expressing rat ACE2 or human ACE2. After 5 h, virus entry was estimated by measuring the amounts of newly synthesized mRNA9 of SARS-CoV. As shown in Fig. 1C, Rat-P15 propagated more efficiently than Frankfurt-1 strain in rat ACE2-expressing CHO cells by more than 10-fold. This result indicates that serial passages of SARS-CoV in rat ACE2-expressing cells efficiently increased its ability to infect rat ACE2-expressing cells.

To examine whether the Rat-P15 strain acquired amino acid substitutions within the S protein during serial passage, the nucleotide sequence of the S gene was determined as described previously (18). Interestingly, the amino acid sequence of the RBD of Rat-P15 was identical to that of the parent Frankfurt-1 strain. In contrast, two amino acid substitutions on the S2 region were found: serine for alanine at amino acid position 811 (A811S) and phenylalanine for serine at position 950 (S950F) (Fig. 1D). Nucleotide sequencing of this region at passages 1, 3, 5, 7, 9, 11, and 13 revealed that A811S substitution occurred after the 11th passage on rat ACE2-expressing cells, whereas the S950F substitution occurred after the 3rd passage (data not shown). This suggests that the two amino acid substitutions had distinct roles in enhancing viral infection in rat ACE2-expressing cells. On the other hand, virus which was passaged 11 times in cells expressing variant rat ACE2 did not have any amino acid substitutions in the S protein. These results suggest that A811S and S950F substitutions were not solely dependent on infection of CHO cells but were triggered by serial passage in rat ACE2-expressing CHO cells.

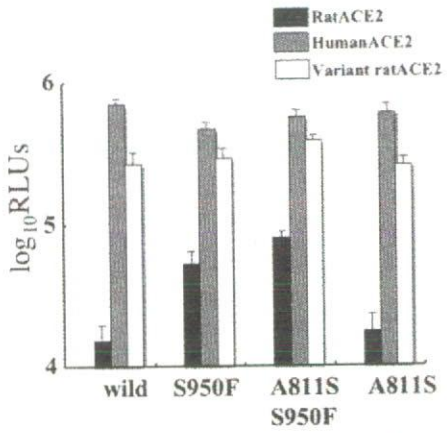

FIG. 2. Analysis of the significance of amino acid substitutions in the S2 region for the efficient entry of the virus into rat ACE2-expressing cells; analysis was done using VSV pseudotypes. VSVΔG*/SEAP-G, in which the VSV glycoprotein gene was replaced with the SEAP (secreted-type *a*lkaline *p*hosphatase) gene, was used for generating VSV pseudotypes bearing the C-terminally truncated S protein of SARS-CoV as described previously (6). BHK cells expressing rat, variant rat, or human ACE2 were inoculated with approximately 10^3 infectious units of VSV-St19 (wild), VSV-St19-A811S (A811S), VSV-St19-S950F (S950F), or VSV-St19-A811S-S950F (A811S S950F). At 18 hpi, SEAP activities of culture supernatants were measured by intensities of chemiluminescence reactions of alkaline phosphatase and are represented as relative luminescence units (RLUs). The VSV pseudotype bearing the S950F substitution rather than the A811S substitution efficiently infected rat ACE2-expressing cells.

In order to analyze the significance of the two amino acid substitutions for the efficient entry of the virus to rat ACE2-expressing cells, a vesicular stomatitis virus (VSV) pseudotyping system (kindly provided by M. A. Whitt) (6) was employed. VSV pseudotypes bearing S protein with a single amino acid substitution (VSV-St19-A811S and VSV-St19-S950F), bearing that with A811S and S950F double amino acid substitutions (VSV-St19-A811S-S950F), and bearing that having the amino acids of the wild type (VSV-St19) were generated. After the expression plasmids encoding rat ACE2, variant rat ACE2, and human ACE2 were transfected to Syrian baby hamster kidney (BHK) cells, each VSV pseudotype was inoculated. All the VSV pseudotypes infected human ACE2- or variant rat ACE2-expressing cells at similar levels (Fig. 2). On the other hand, VSV-St19-A811S-S950F infected rat ACE2-expressing cells more efficiently than VSV-St19. Interestingly, VSV-St19-S950F, which carries the S protein with the S950F substitution, infected rat ACE2-expressing cells more efficiently than did VSV-St19-A811S with the A811S substitution or VSV-St19. This indicates that the S950F substitution has a significant role in the efficient entry mediated by rat ACE2.

It has been shown that a single amino acid substitution in the S2 region affects the maturation of the glycosylation process of SARS-CoV S protein (1). Several lines of evidence indicate that glycosylation of viral envelope proteins is a molecular determinant for virus replication and infectivity (22, 24, 25, 26). Therefore, the wild-type and mutant S proteins were subjected to Western blotting to investigate the effect of the amino acid substitutions on the glycosylation of the S protein. The mutant S protein with a single A811S substitution (S-A811S), as well as the wild-type S protein (S-wt), migrated somewhat more slowly than those with a single S950F substitution (S-S950F) or with A811S and S950F substitutions (S-A811S-S950F) (Fig. 3). When S proteins were digested with endo-H, all mutant proteins migrated with molecular masses comparable to those of undigested controls. In contrast, when the expressed S proteins were digested with PNGase-F, all the mutant proteins showed migration patterns similar to that of the wild-type S protein. This indicates that differences in migration patterns of S-S950F and S-A811S-S950F were due to altered attachment of complex oligosaccharides during maturation. Our results indicate that S950F substitution affected N-linked glycosylation of the S protein and suggest that these differences are well correlated with the increased efficiency of SARS-CoV infection of rat ACE2-expressing cells.

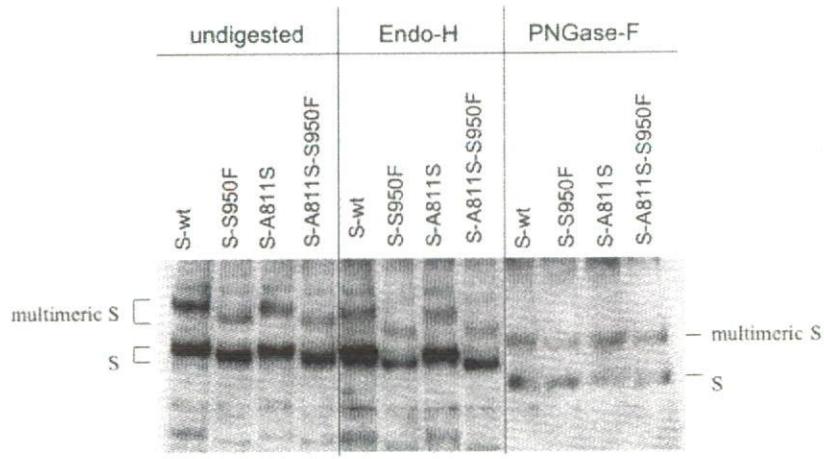

FIG. 3. Analysis of N-linked glycosylation of S proteins. SARS-CoV S proteins with the wild-type sequence (S-wt), an S950F substitution (S-S950F), an A811S substitution (S-A811S), and A811S and S950F substitutions (S-A811S-S950F) that were expressed in 293T cells were treated with endo-H or PNGase-F and then subjected along with undigested samples to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The S protein was detected by Western blotting using a rabbit antibody specific for the S2 region (6).

In contrast to our findings that amino acid substitutions were found in the S2 region, a substitution of tyrosine by serine located in the RBD was detected in the in vivo-adapted SARS-CoV (18). The difference between in vivo and in vitro passage may be attributed to replication sites; the substitution on the RBD seems to be responsible for the efficient replication of the virus on the alveolar area, where ACE2 is expressed at a low level (18), whereas virus replicated in CHO cells where ACE2 was abundantly expressed by transfection of expression plasmids. Alternatively, it seems likely that innate immune responses in rats could select a particular SARS-CoV strain adapted to rats. In summary, SARS-CoV adaptation to a particular animal species can be induced by amino acid substitutions in the RBD within the S1 region but also by those in the S2 region.

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Short communication

Rapid determination of viral RNA sequences in mosquitoes collected in the field

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Abstract

A method for rapid determination of viral RNA sequences (RDV) was applied to homogenates of *Aedes aegypti* collected in Thailand in an area in which dengue fever (dengue hemorrhagic fever) is endemic, using the mosquito cell line C6/36. Nucleic acid sequences of dengue virus type 4 and cell fusing agent virus were detected. This RDV method has the potential to become a standard method for detection of both known and newly emerging, unknown mosquito-borne viruses.

Keywords: Rapid determination; Dengue virus type 4; Cell fusing agent virus; *Aedes aegypti*

Dengue viruses (DENV) cause dengue fever, the most important mosquito-borne viral disease, and these viruses pose a major public health problem in tropical and subtropical areas (Guzman and Kouri, 2002). *Aedes aegypti* is the primary and most effective epidemic vector of DENV (Gubler, 1998). Virological surveillance of mosquitoes naturally infected with DENV, using a reverse transcriptase-polymerase chain reaction (RT-PCR), is thought to be a fast and effective predictive method for detecting dengue outbreaks (Urdaneta et al., 2005). However, it is possible for mosquitoes to also carry viruses that are unknown or that cannot currently be identified by RT-PCR based on known viral nucleic acid sequences. Therefore, a system for rapid nucleic acid sequence determination is necessary to identify newly emerging mosquito-borne viruses.

Recently, a method was developed to determine rapidly the RNA of viruses (RDV) that can determine the nucleotide sequence of viral RNA without a specific primer (Mizutani et al., 2007). It was also possible to detect mosquito-borne viruses, such as West Nile virus, Japanese encephalitis virus, and DENV-2, from culture supernatants (Mizutani et al., 2007). The RDV method can detect at least 10^4 copies of *in vitro* synthesized RNA (unpublished data). In this study, the RDV method was used as a virological surveillance tool on *Ae. aegypti* collected in the field in Thailand.

Ae. aegypti specimens (93 adult females) were collected around houses of patients diagnosed clinically with dengue fever in Jomthong District, Bangkok, Thailand, in July 2006. Each mosquito was placed in a 0.5 ml tube and was homogenated

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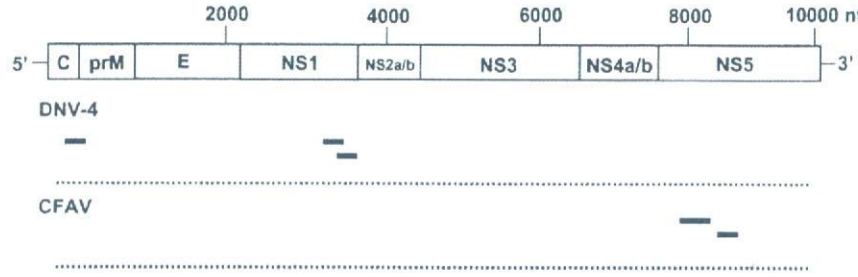

Fig. 1. Location of PCR fragments of viral cDNAs amplified using the RDV method. Amplified PCR fragments were directly sequenced and mapped in the flavivirus genome.

using a pellet mixer in 100 μl of minimum essential medium (MEM) with 4% fetal bovine serum (FBS). The homogenates were centrifuged at 2500 rpm for 10 min at 4 °C, and 50 μl of the supernatant was used. RT-PCR was performed using the OneStep RT-PCR enzyme mix (Gibco-BRL, Cergy Pontoise, France) and primers designed to detect all four serotypes of DENV. Eight of the 93 homogenates were DENV-positive. Four DENV-positive homogenates numbered 1, 2, 3 and 4 were filtrated using a Millex-GX filter (Millipore Corp., Bedford, Massachusetts), and the filtrate was added to a C6/36 cell culture (7×10^5 cells) in a 24-well plate. Total volume was brought to 1 ml per well by adding MEM with 2% FBS, and was incubated at 28 °C in a CO_2 incubator. After 9 days, cytopathic effects were observed in the cells exposed to DENV-positive homogenate. The culture supernatants were collected and centrifuged at 2500 rpm for 10 min at 4 °C. Then, 50 μl of the supernatant was used for cDNA synthesis.

The four cDNAs were mixed for the following RDV method. The protocol for the RDV method is described in detail elsewhere (Mizutani et al., 2007). The nucleic acid sequences from 16 fragments were used to search for homological sequences using BlastN. Three fragments were found to be highly homologous with DENV-4, and two fragments were found to be homologous with cell fusing agent virus (CFAV) (Fig. 1). CFAV belongs to flavivirus that does not have a vertebrate host (Stollar and Thomas, 1975). CFAV replicates in *Aedes* mosquitoes and in mosquito cells. No antigenic cross-reaction was found between CFAV and other member of flavivirus (Cammisa-Parks et al., 1992). The nucleotide sequence data are available in the DDBJ/EMBL/GenBank databases under accession nos. AB300619–AB300621 (DENV-4) and AB300622–AB300623 (CFAV). This result shows that nucleic acid sequences of DENV-4 and CFAV can be detected using the RDV method without using specific primers. To determine which homogenates contained these viruses, the four cDNAs were pre-amplified individually using a GenomiPhi V2 kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) according to manufacturer's protocol. PCR was performed using KOD-Plus (Toyobo Co. Ltd., Osaka, Japan). Primers for detecting DENV-2 and DENV-4 are described by Morita et al. (1994). Three primer sets for detecting CFAV were designed to be at the NS4A region of CFAV based on accession number NC_001564. DENV-4, but not DENV-2, was detected in all four of the cDNA samples, whereas CFAV was only detected in the cDNA of homogenate 1, but not in homogenates 2, 3, and 4 (data not shown), indicating that both DENV-4 and CFAV co-infected the mosquito of homogenate 1. Integration of part of the CFAV genome in C6/36 genomic DNA has been reported (Crochu et al., 2004). However, CFAV was not detected from RNA and DNA extracted from two different C6/36 cell stocks using RT-PCR (data not shown). Although it cannot be ruled out that the CFAV genome was integrated in the genome of the C6/36 cells below detection level, these results strongly suggest that CFAV nucleic acid sequences found by the RDV method originated from the mosquito of homogenate 1.

It was demonstrated that the RDV method is able to detect DENV and CFAV without using specific primers for amplification. There have been a number of mosquito-borne outbreaks of flaviviruses recently, and birds are also important vectors for viruses such as West Nile virus. Recently, direct determination of avian viral RNA sequences was demonstrated in allantoic fluids inoculated with a test specimen using the RDV method (Sakai et al., 2007). The RDV method therefore has the potential to become a standard method for the detection of both known and newly emerging, unknown avian-borne and mosquito-borne viruses.

Acknowledgments

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Short Communication

Simultaneous Detection of the Genus *Brucella* by Combinatorial PCR

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SUMMARY: We have developed a combinatorial polymerase chain reaction (PCR) procedure to identify four major species of the genus *Brucella* simultaneously. Four pairs of primers targeting the genes encoding a cell surface protein (*BCSP31*) and outer membrane proteins (*omp2b*, *omp2a* and *omp31*) were prepared. PCR using these primers gave rise to specific patterns of amplification for each *Brucella* spp. examined in this study. *B. abortus* could be identified when fragments of *BCSP31* and *omp2b*/*2a* were amplified by *B. abortus*-specific primers. *B. melitensis* could be identified by the amplification of fragments of *BCSP31*, *omp2b*/*2a* and *omp31* using pair of primers B4/B5, JRF/JPR-ab and *omp31*. Identification of *B. canis* could be achieved when the amplicons of *omp2b*/*2a* were detected by *B. canis*-specific primers, as could the identification of *BCSP31* and *omp31*. If specific amplifications occurred using all pairs of primers, the strain was identified as *B. suis*. Combinatorial PCR reported here thus appeared to be an ideal method of identifying *Brucella* spp., the causative pathogen of human brucellosis.

Brucellosis, a zoonosis caused by bacteria belonging to the genus *Brucella*, is endemic in various parts of the world, especially in countries of the Mediterranean region, Asia, Africa and South America (1-3). Among the species of the genus *Brucella*, the four major causative agents of human brucellosis are *B. melitensis*, *B. abortus*, *B. suis* and *B. canis*, although their natural hosts are usually confined to goats and sheep, cattle, pigs and dogs, respectively (1-3). Moreover, some species of the genus *Brucella* are considered to be potential agents for bioterrorism (4).

Microbiological, serological and molecular techniques are commonly used for the diagnosis of brucellosis (1,2,5). Microbiological tests such as the isolation of bacteria from host tissues or blood cultures followed by bacteriological characterization remain important, although they are tedious and time-consuming (2,5). The most widely used serological tests, i.e., tube agglutination tests using inactivated *B. abortus* or *B. canis* as antigens, show some degree of cross-reaction with other bacterial strains (1,2). Moreover, it is difficult to serologically distinguish the species within the genus *Brucella* using the tube agglutination test (1,2).

Among molecular techniques, polymerase chain reaction (PCR) is one of the most useful tools for the diagnosis of brucellosis. It has been reported that identification of the genus *Brucella*, but not of the species within the genus, can be performed by PCR using primers targeting highly conserved regions such as the *BCSP31* (6) or 16S-rRNA (7). As regards the differentiation of species and/or biovars of *Brucella* within the genus, several laboratories have reported PCR procedures using highly specific primers and/or stringent assay conditions. For example, it was reported that *B. abortus* could be distinguished from *B. melitensis* by species-specific PCR targeting IS711 using primers designed based on the nucleotide sequences of *B. abortus* (8,9). *B. suis* could also be discriminated from *B. abortus* using primer pairs designed according to *B. suis*-specific sequences (10). Furthermore, identification of *B. canis* could be accomplished by using specific primers designed to amplify *virB2* (11).

In Japan, the prevalence of brucellosis is quite low, but cases of *B. melitensis* infection have recently been reported (12,13). It remains possible that some people in Japan currently suffer from brucellosis, since canine brucellosis caused by *B. canis* still exists in this country. Therefore, a reliable diagnostic system capable of distinguishing between the four species of the genus *Brucella*, including *B. canis*, remains necessary. In the present study, we attempted to develop a PCR approach that could be used to identify the four major species of the genus *Brucella* simultaneously using newly designed primers.

Here, we used 11 strains belonging to the genus *Brucella* and 23 strains of non-*Brucella* bacteria (Table 1). *Brucella* strains were cultured on sheep blood agar plates and the DNA was isolated using SepaGene (Sanko Junyaku, Tokyo, Japan) according to the protocol supplied by the manufacturer. DNA from non-*Brucella* strains was also prepared.

Isolated DNA was amplified using puReTaq Ready-To-Go PCR Beads (GE Healthcare Bio-Science Corp., Piscataway, N.J., USA) by PCR consisting of initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 65°C for 1 min and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min.

The primers designed and used for the simultaneous identification of four major *Brucella* spp. are listed in Table 2. The results are shown in Fig. 1 and summarized in Table 1. A pair of primers, B4/B5, was previously reported to amplify a 224-bp DNA fragment from a gene encoding a 31-kDa cell surface protein (*BCSP31*) that is well conserved in all *Brucella* spp. (M20404) (6). We have confirmed that this pair of primers is specific for the genus *Brucella*, since no PCR product was detected when DNA from bacteria other than *Brucella* spp. was used as templates (Table 1). The gene encoding *Brucella* major outer membrane protein 2 (*omp2*) has two related regions, *omp2b* and *omp2a*, and these two regions are 85% homologous and oriented in opposite directions (U26438) (14). Leal-Klevezas et al. reported that a 193-bp fragment could be amplified with a pair of primers, JPF/JPR, from *B. abortus*, *B. melitensis* and *B. suis*, but not from

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Table 1. Bacterial strains used in this study and the results of PCR

Species	Strain	*BCSP31*	*omp2b*		*omp2a*		*omp31*
		B4/B5	JPF/JPR-ab	JPF/JPR-ca	JPF/JPR-ab	JPF/JPR-ca	1S/1AS
Brucella abortus	544[1]	+	+	−	+	−	−
Brucella abortus	Takanashi[1]	+	+	−	+	−	−
Brucella abortus	125[2]	+	+	−	+	−	−
Brucella melitensis	16M[1]	+	+	−	+	−	+
Brucella melitensis	HagiwaraB1[1]	+	+	−	+	−	+
Brucella melitensis	TWCC40430[3]	+	+	−	+	−	+
Brucella melitensis	H17-298[4]	+	+	−	+	−	+
Brucella suis	1330[1]	+	+	−	−	+	+
Brucella suis	S-13[1]	+	+	−	−	+	+
Brucella canis	QE13[1]	+	−	+	−	+	+
Brucella canis	Shizuoka03[5]	+	−	+	−	+	+
Yersinia pestis	Yreka	−	−	−	−	−	−
Yersinia pestis	A1122	−	−	−	−	−	−
Yersinia enterocolitica	Pa2369 (O3)	−	−	−	−	−	−
Yersinia enterocolitica	Pa9571 (O5)	−	−	−	−	−	−
Yersinia enterocolitica	Pa12986 (O8)	−	−	−	−	−	−
Yersinia enterocolitica	Pa177 (O9)	−	−	−	−	−	−
Yersinia pseudotuberculosis	319	−	−	−	−	−	−
Bacillus anthracis	PAII	−	−	−	−	−	−
Bacillus cereus	NBRC3466	−	−	−	−	−	−
Bacillus subtilis	3	−	−	−	−	−	−
Francisella tularensis	LVS	−	−	−	−	−	−
Coxiella burnetii	Nine Mile	−	−	−	−	−	−
Eschericia coli	DH5 alpha	−	−	−	−	−	−
Haemofilus influenzae	Type B	−	−	−	−	−	−
Klebsiella pneumoniae	ATCC13883	−	−	−	−	−	−
Listeria monocytogenes	ATCC15315	−	−	−	−	−	−
Mycobacterium tuberculosis	ATCC27294	−	−	−	−	−	−
Pasteurella aerogenes	ATCC27883	−	−	−	−	−	−
Pasteurella multocida	ATCC12947	−	−	−	−	−	−
Staphylococcus aureus	ATCC29247	−	−	−	−	−	−
Streptobacillus moniliformis	ATCC14647	−	−	−	−	−	−
Ochrobactrum anthropi	ATCC49187	−	−	−	−	−	−
Ochrobactrum anthropi	ATCC49687	−	−	−	−	−	−

[1]: Bacterial strains were supplied from National Institute of Animal Health, Tsukuba, Ibaraki, Japan.
[2]: Heat-inactivated bacteria, which was commercially available, was obtained from National Agriculture and Food Research Organization, Tsukuba, Ibaraki, Japan as an antigen for a tube agglutination test.
[3]: A new isolate from blood of an imported brucellosis patient was supplied from Tokyo Women's Medical University, Tokyo, Japan.
[4]: A new isolate from blood of an imported brucellosis patient was supplied from Tokyo Metropolitan Institute of Public Health, Tokyo, Japan.
[5]: A new isolate from a piece of liver of an aborted puppy was isolated in our laboratory.

Table 2. Primers designated in this study

Target gene	Primer name	Sequence	Target length	GenBank accession	Location
BCSP31	B4 (S)[1]	5'-Tgg CTC ggT TgC CAA TAT CAA	224 bp	M20404	789-809
	B5 (AS)[1]	5'-CgC gCT TgC CTT TCA ggT CTg		M20404	1012-992
omp2	JPF (S)[2]	5'-gCg CTC Agg CTg CCg ACg CAA		U26438	2110-2130
	JPR-ab (AS)	5'-CAT TgC ggT Cgg TAC Cgg Ag	186 bp	U26438	2295-2276
	JPR-ca (AS)	5'-CCT TTA CgA TCC gAg CCg gTA	187 bp	U26439	2296-2276
omp31	1S (S)	5'-gTT CgC TCg ACg TAA CAg CTg	249 bp	AF366073	218-238
	1AS (AS)	5'-gAC CgC Cgg TAC CAT AAA CCA		AF366073	446-466

Primers 1) and 2) were prepared according to reference 6 and 8, respectively. Others were newly designated in this study.

B. canis (15). In this study, we designed two novel antisense primers, JPR-ab and JPR-ca, which are specific for *B. abortus* (U26438) and *B. canis* (U26439), respectively. In *B. abortus* and *B. melitensis*, it was observed that 186-bp fragments from both *omp2b* and *omp2a* regions were amplified by PCR with the pair of primers, JPF/JPR-ab. In contrast, since the nucleotide sequences of target regions of *B. canis* differ from those of *B. abortus* and *B. melitensis*, the *B. canis* fragments *omp2b* and *omp2a* were amplified only when the JPR-ca primer was used together with the JPF primer. On the other hand, since the sequences of amplicons of *omp2b* and *omp2a* of *B. suis* (U26443) are identical to those of *B. abortus* and

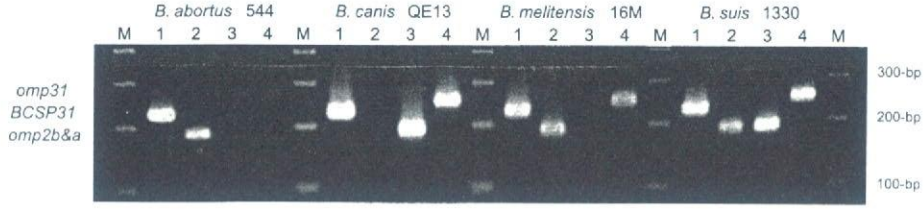

Fig.1. Detection patterns of four major biovars of *Brucella* spp. by four pairs of primers. Lane 1, BA/B5; 2, JPF/JPR-ab; 3, JPF/JPR-ca; 4, 1S/1AS; M, Size marker.

B. canis, respectively, fragments were obtained by PCR using either the JPF/JPR-ab or JPF/JPR-ca primers. As shown in Fig. 1, PCR using a pair of primers, JPF/JPR-ab, amplified a 186-bp fragment from *B. abortus*, *B. melitensis* and *B. suis*, but not from *B. canis*, while the primers JPF/JPR2-ca amplified a 187-bp fragment from *B. canis* and *B. suis*. A pair of primers 1S/1AS was designated to amplify a 249-bp fragment from the *omp31* gene encoding another *Brucella* outer membrane protein. However, due to the presence of a large deletion in the *omp31* gene of *B. abortus* (14,16), primers 1S/1AS did not amplify the fragment from *B. abortus* (Table 1).

These results demonstrated that four species of *Brucella* could be successfully identified by combinatorial PCR using four sets of primers (Fig. 1). *B. abortus* could be identified when the amplification of fragments of *omp2b* and *omp2a* by *B. abortus*-specific primers (JRF/JPR-ab) took place, moreover, *B. abortus* could also be identified based on the amplification of *BCSP31* by B4/B5 primers. *B. melitensis* could be identified by the amplification of fragments of *BCSP31* and *omp31* as well as *omp2b* and *omp2a* by a pair of primers, JRF/JPR-ab, and *omp31*. In contrast, identification of *B. canis* could be achieved if *BCSP31* and *omp31* were amplified and if the amplicons of *omp2b* and *omp2a* were detected by *B. canis*-specific primers (JRF/JPR-ca), but not by *B. abortus*-specific primers (JRF/JPR-ab). In cases when specific amplifications occurred using all pairs of primers, the strain was identified as *B. suis*. In this study, we included 23 bacteria belonging to genera other than *Brucella* spp. Since the PCR series reported here did not amplify any fragments from these 23 non-*Brucella* bacteria, this method appears to be highly specific for the genus *Brucella*. Moreover, this PCR also amplified specific sequences from mouse tissue homogenates and blood experimentally spiked with *B. abortus* or *B. canis* (data not shown). The PCR detection limit was observed to be approximately 1 pg of DNA (data not shown).

Since multiplex PCR has been used for the simultaneous detection of several pathogens, we also attempted to establish a multiplex PCR for the detection of *Brucella* spp. However, the detection limit of that multiplex approach was inferior to that of the PCR reported here, most likely due to the competitive consumption of ingredients among amplicons (data not shown). Additionally, multiplex PCR using the primer pairs, B4/B5, JPF/JPR-ab and 1S/1AS, did distinguish *B. abortus* and *B. canis* from other *Brucella* spp., but *B. melitensis* could not be differentiated from *B. suis*. In contrast, using the primer pairs, B4/B5, JPF/JPR-ca and 1S/1AS, we were able to distinguish *B. abortus* and *B. melitensis* from the other species by multiplex PCR, although *B. canis* and *B. suis* could not be differentiated in this manner (Table 1). From these results, we concluded that it was more practical to perform combinatorial PCR rather than a multiplex PCR to identify the genus *Brucella* at the species level.

Although we tested a limited number of biovars belonging to each *Brucella* spp., it appears likely that the method reported here will enable the reliable identification of the four major species of the genus *Brucella* which infect human beings.

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IASR

ブルセラ症（1999年4月～2007年3月31日現在）

(Vol.28 p 227-228:2007年8月号)

ブルセラ症(brucellosis)はブルセラ属菌(Genus *Brucella*)による人獣共通感染症である。ヒトに感染する菌種は病原性の強い順に、*B. melitensis*（自然宿主:ヤギ、ヒツジ）、*B. suis*（ブタ）、*B. abortus*（ウシ）である。これら家畜の持つブルセラ菌のヒトへの感染は、感染動物の加熱(殺菌)処理していない生乳およびそれから作ったチーズ、食肉の喫食や、死体・流産時の汚物・汚染物などとの接触や、それらからのエアロゾルの吸入による。授乳、性交などによるヒトーヒト感染もありうるが、極めてまれである。潜伏期は通常1～3週間であるが、時に数カ月に及ぶこともある。軽症の場合、単なる感冒様症状のこともある。通常、症状は他の熱性疾患と似ているが、筋・骨格系への影響が強く、全身的な疼痛・倦怠感や、間欠熱・波状熱といった特徴的な熱型を示すこともある。これらの症状は数週間～数カ月、数年に及ぶこともある。*B. canis*（自然宿主:イヌ)もヒトに感染することがあるが一般に症状は軽く、気がつかないケースも多い。感染イヌは流産を起こすが、その流産胎子、胎盤、汚物や、尿、精液などへの接触により感染する。

本疾患は世界中で発生している。特に家畜での対策が不十分な地域では、年間数百～数千症例のヒト患者が報告されているが、実際の患者数はその10～25倍以上と推定されている。地域的には、特に西アジア、中東、地中海沿岸、アフリカ、中南米、カリブ海諸国などに多い。日本では家畜対策(摘発・淘汰)が功を奏し、清浄化していると考えられ、従って家畜から感染する可能性は低い。ただし、イヌでは2～5%前後が*B. canis*の感染歴を持つとされている。

わが国では従来、本疾患は届出の対象ではなかったため、発生状況は正確に把握されていなかった。しかし、1999年4月1日施行の感染症法で4類感染症に指定され、診断したすべての医師に届出が義務づけられた。それ以降、2007年3月31日現在までに届出は8例みられているが、2005年2例、2006年5例と、近年に集中している(表)。これは実際に患者数が増加したことよりも、むしろ診断の際にブルセラ症が考慮されるようになったためと考えられる。

国外を推定感染地域とする4例のうち、血液培養により菌が分離同定されて、*B. melitensis*感染が確定された2例(表中#2、4)は、いずれも海外で感染したものである。1例はシリアでの羊肉の摂食によると考えられ(IASR 26: 273-274, 2005参照)、もう1例はエジプトでの環境からのエアロゾル吸入による可能性が最も疑われている(IASR, 27: 125-126, 2006参照)。*B. abortus*感染が確定された1例(表中#6)は海外で感染・発症し、治療を受けたが、国内で再燃したと考えられており、感染原因としてエジプトでのミルクの摂取が推定されている。このように、本疾患は輸入感染症として注意する必要がある。

国内を推定感染地域とする3例は、いずれも*B. canis*に対する抗体が検出されているが、3例ともに明らかなイヌとの接触歴は認められなかった。

ブルセラ症の症状には特徴的なものがなく、診断には血清抗体測定や菌分離などの病原診断が欠かせない。血清診断は通常、*B. abortus*や*B. canis*を抗原とした試験管内凝集反応が行われ、民間の臨床検査機関でも可能であるが、凝集抗体価がそれぞれ1:40、1:160以上の時に陽性と判断される(従来、抗原がいずれであっても160倍以上の抗体価をもって届出の対象とされていたが、2007年4月に*B. abortus*については40倍以上を対象とすることに変更された)。*B. melitensis*、*B. suis*感染が疑われるときでも、*B. abortus*を抗原とした抗体の検出を行う。菌種の特定には菌分離が必要であり、血液や骨髄の培養が行われるが、抗菌薬がすでに投与されていて分離できないことが多い。これまでの報告でも、特に国内での感染が疑われる3例ではすべて菌が分離されておらず、病原診断は凝集反応陽性によりなされている。しかも、1例(表中#3)を除き、単血清での陽性結果で診断されているが、血清抗体のみで確定診断するにはペア血清を用いることが望ましい。また、PCR法による病原体遺伝子診断も可能であり、国立感染症研究所獣医科学部に依頼が可能である。

国立感染症研究所獣医科学部第一室　今岡浩一
国立感染症研究所感染症情報センター第二室

表 ブルセラ症の報告症例（1999.4.1～2007.3.31）

症例番号	感染推定年月	発病年月	診断年月	性	年齢	報告都道府県	推定感染地	推定感染経路	症　状	血清抗体検査 *	菌分離
1	記載なし	記載なし	2002.1	女	40代	東京都	不明	ペットの犬	発熱、食欲不振	BA(-)、BC(+)	(-)
2	記載なし	2005.6	2005.6	女	30代	東京都	シリア	経口（羊肉）	発熱、皮疹、脾腫、腹部リンパ節腫大、関節痛	BA(+)、BC(+)	*B. melitensis*
3	記載なし	2005.10	2005.12	男	10代	長野県	国内（都道府県名情報なし）	不明	発熱、筋肉痛、腹痛	BA(-)、BC(+)	(-)
4	記載なし	2006.2	2006.2	男	50代	東京都	エジプト	不明（エアロゾル吸入疑い）	発熱、頭痛、肝脾腫	BA(+)、BC(+)	*B. melitensis*
5	2006.3	2006.5	2006.6	女	20代	長野県	イタリア	不明	発熱、筋肉痛	BA(-)、BC(+)	(-)
6 **	2005.9	2006.7	2006.7	女	20代	北海道	エジプト	経口（ミルク）	発熱、頭痛	BA(+)、BC(-)	*B. abortus*
7	2006.1	2006.5	2006.9	女	60代	長野県	長野県	不明	発熱、脾腫	BA(-)、BC(+)	(-)
8	2006.8	2006.9	2006.10	女	70代	宮城県	宮城県	不明	発熱、中枢神経症状	BA(-)、BC(+)	(-)

*：試験管内凝集反応。抗原として*B. abortus* (BA) または*B. canis* (BC) を使用

**：過去(2005.9)にエジプトにて発症、治療。今回は再燃と思われる

LASR

Capnocytophaga canimorsus が分離された、敗血症・多臓器不全で搬送され救命し得たイヌ咬傷の1例

(Vol.28 p 299-300:2007年10月号)

2006年11月、東京女子医大東医療センター救命救急センターに、多臓器不全の診断で75歳の女性が搬送された。ペットの犬に手首を咬まれた2日後に呼吸困難で近医を受診しており、イヌ咬傷に起因する敗血症と診断し、集中治療を行った。経過良好で、第14病日に軽快退院となったが、血液培養より、*Capnocytophaga canimorsus* が分離された。犬や猫の口腔内常在菌であるが、本邦における敗血症の報告例は稀である。

症例:75歳、女性。
主訴:呼吸困難。
既往歴・家族歴:特記なし。
現病歴:2006年11月2日飼い犬に左手首を咬まれるが、自分で消毒し放置。11月4日呼吸困難が出現し、近医を受診。意識は清明であったが、動脈血ガス検査にて、PO_2 56mmHg、PCO_2 31mmHgと低酸素血症を認める。血液検査にて、白血球 18,000/μl、CRP 5.04mg/dlと高値。血小板 1.5万/μlと著明に減少。さらにBUN 59mg/dl、Crt 2.43mg/dlと腎機能障害を認める。以上より、敗血症の疑い、DIC(播種性血管内凝固症候群)、多臓器不全の診断にて、集中治療を目的に当センターに転送。

搬送時現症:意識レベルI-1、血圧94mmHg 触診、脈拍98/分、呼吸数24/分、体温39.0℃、O_2SAT測定不能、全身にチアノーゼ著明、左手首に2カ所のイヌの歯型と発赤・腫脹あり。

搬送時検査成績:(表1)。

臨床経過(図1):呼吸苦が顕著であったため、鎮静し人工呼吸器管理とし、第1病日エンドトキシン吸着・持続血液濾過透析を施行し、抗菌薬 (IPM/CS 1g、CLDM 1,200mg)・ガンマグロブリン・FOY・AT-III製剤投与による治療を開始した。第3病日CRP 31mg/dlまで増悪したが、以後は改善傾向となり、血小板も20単位×2日間の血小板輸血後、第4病日より増加傾向となった。以後順調に経過し、第6病日 人工呼吸器より離脱。第14病日CRP 3.7mg/dlまで低下したため抗菌薬は中止とし、同日退院とした。入院中、動脈血液培養よりグラム陰性桿菌が検出されたが同定に至らず、国立感染症研究所に依頼した。国立感染症研究所において、イヌ咬傷原因菌の1つである*Capnocytophaga* 属菌の16S rRNA遺伝子特異的PCRを実施した結果、*C. canimorsus* 特異的遺伝子が検出された。また、併せて実施した生化学的性状検査も*C. canimorsus* の性状を示していた。以上のことから、本症例の原因菌は*C. canimorsus* と判明した。

考察:*C. canimorsus* はイヌの口腔内常在菌であるが、イヌ咬傷後の敗血症の原因菌として欧米では死亡例も多数報告される1)。本邦での報告は稀で、文献検索では、いずれも敗血症を合併し死亡した、ペットの猫に咬まれ受傷した95歳の女性2)と、同じくペットの猫に引っ掻かれ受傷した63歳の男性3)の2例のみであったが、菊池らは『本邦では血液培養の頻度が少なく、本菌による敗血症が見逃されている可能性が高い』と指摘している2)。また、欧米の報告では、高齢者・易感染者(糖尿病・アルコール中毒・脾臓摘出術後など)に重症例が多い1,4)。本症例の危険因子は'75歳の年齢'だけであったが、集中治療が遅れれば不幸な転帰となったことは十分に想像し得る。既往に特記のない健常者で、管理された飼い犬・猫であっても、咬傷・掻傷の際は早期に医療機関を受診するよう注意するべきで、医療側からの啓発も重要である。抗菌薬の選択については、一般に犬・猫による咬・掻傷では、起炎菌として黄色ブドウ球菌・連鎖球菌・*Pasteurella* 属・種々の嫌気性菌が想定され、Amoxicillin-clavulanateまたはClindamycin + ST合剤の内服が第一選択となる。入院治療では、Ampicillin-sulbactamまたはClindamycin + Ciprofloxacin hydrochlorideが選択される5)。*C. canimorsus* はPCG(ペニシリンG)が第一選択であるが6)、上記の抗菌薬にも感受性を示し、本症例で使用したImipenem, Clindamycinもほぼ妥当であったと考える。

結語：本邦では非常に稀である*C. canimorsus* による敗血症・多臓器不全の症例を経験した。犬・猫の口腔内常在菌であるが、敗血症の原因菌となる場合もあり、ペットによる咬傷・掻傷の際は、軽症であっても医療機関を受診し、適切な局所の消毒・抗菌薬投与を受けるべきである。同時に医療側もより広い啓発への自覚が必要である。示唆に富む貴重な症例と考え報告した。

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表１．当センター搬送時検査成績

Hematology

WBC	125×10³/mm³
RBC	395×10⁴/mm³
Hb	12.9 g/dl
Plt.	1.2×10⁴/mm³

Coaguration test

PT	17.2 sec.
INR	2.05
APTT	62.3 sec.
FIB	186 mg/dl
HPT	37.7 %
ATⅢ	37 %
FDP	212.8 μg/ml

Blood chemistry

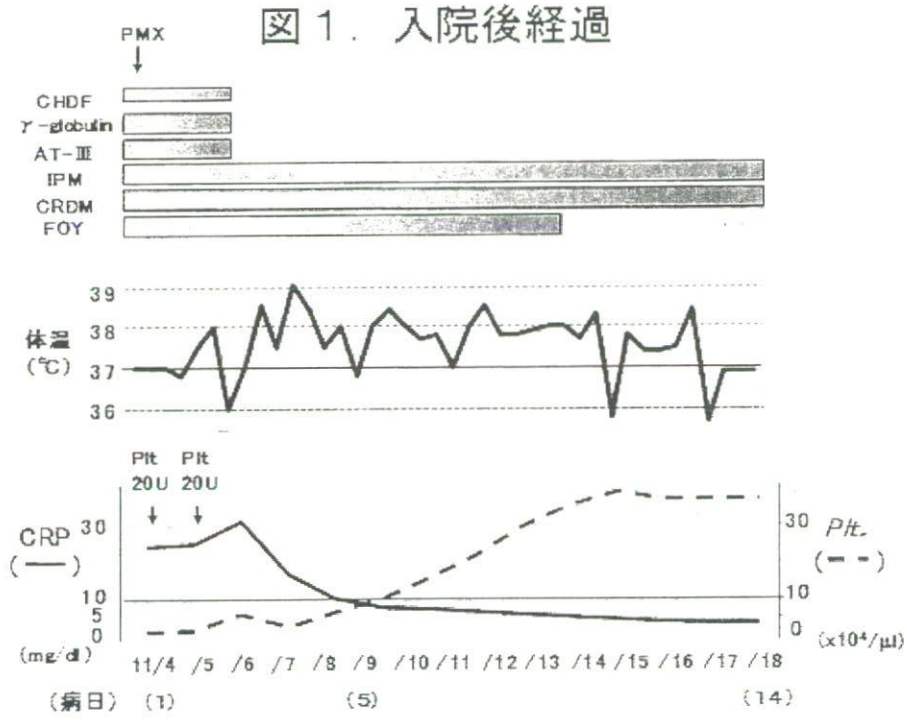
TP	5.3 g/dl
Alb	3.0 g/dl
AST	105 IU/l
ALT	38 IU/l
LDH	599 IU/l
ALP	420 IU/l
γGTP	71 IU/l
Amylase	96 IU/l
CPK	1337 IU/l
T-Bil	1.9 mg/dl
BUN	61.9 mg/dl
Crt.	2.53 mg/dl

Na	137 mEq/l
K	4.5 mEq/l
Cl	100 mEq/l

Serological test

CRP	24.36 IU/l
HBsAg	(−)
HCV-Ab	(−)

図１．入院後経過



特集 意外と知らない!? 感染症

4 犬にかまれた！ どうしよう…

～パスツレラ症，カプノサイトファーガ症，狂犬病～

国立感染症研究所獣医科学部 今岡浩一（いまおかこういち）

今，日本では1,200万～1,300万匹の犬がペットとして飼われています（日本ペットフード工業会調べ）。人のもっとも身近な伴侶動物である犬は，チュウゴクオオカミを祖先として2万～1万5千年前と，ほかの動物に比べもっとも早く家畜化された動物だといわれています。そのため，人との付き合いも古く，その習性や病気についてもすでによく知っていると思いがちです。一般的に，動物から人への病原体の伝播は，その距離が近いほど容易になります。したがって，関係が親密な犬は特に注意が必要な動物でもあるわけです。

犬咬傷の実態は？

たとえ注意していても，何かのきっかけでかまれてしまうことがあります。環境省の調査によると国内の犬咬傷事故は，年間6,300件ほどです（2001年）。米国では，日本の約5倍（6,000万匹ほど）の犬が飼われていますが，年間470万人が犬にかまれ，80万人が医療機関を受診し，6,000人が入院しているという調査報告があります。これらの患者がすべて感染症を発症しているわけではありませんが，環境省の調査報告に入っていない小さな事故もあるので，日本でも実際はかなり多くの咬傷事故・感染者が発生していると推定されます。

また，同じく米国の調査では，犬にかまれた人の半数が子ども（14歳

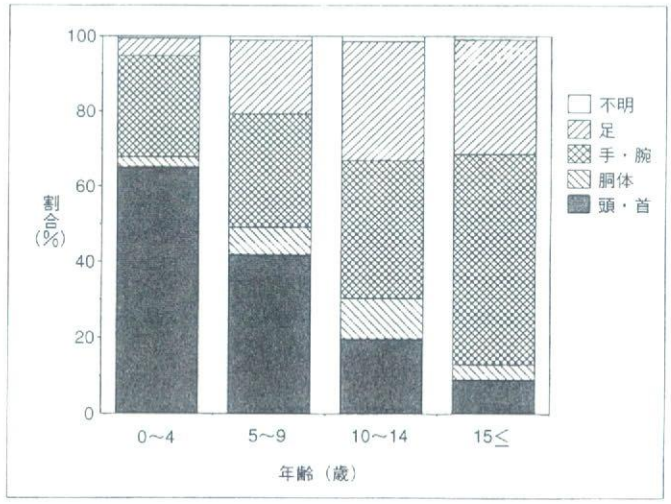


図1 人の年齢と犬による咬傷部位 （文献[1]より引用）

著者プロフィール 1990年東京大学大学院博士課程修了。国立公衆衛生院（現国立保健医療科学院）を経て，2002年より現職。獣医師。農学博士。専門は人獣共通感染症学。おもな著書に「動物由来感染症―その診断と対策―」（真興交易，2003年）など。

1344-3151/07/¥50/頁/JCLS

以下）で，特に5～9歳では男児が多くなっていました。かまれる部位は，図1に示したように年齢が低くなるほど，頭・首をかまれる割合が高く，年齢が上がるとともに四肢（手足）をかまれる割合が上がっていました。日本の場合は，飼い犬が飼い主以外の成人をかむケースが多い（約70%）との報告もあります。季節的には，犬の発情期や人が薄着になる関係からか，春から夏に多くなっているようです。

犬にかまれることで感染する病気は？

犬にかまれると単に外傷だけではなく，感染症の病原体に感染することがあります。では，咬傷に伴う代表的な感染症にはどのようなものがあるのでしょうか。

パスツレラ症：犬咬傷による感染症のうち，約半数から分離されるもっとも代表的なものです。50～70%程度の犬が，口腔内常在菌としてパスツレラ・マルトシダなどのパスツレラ属菌の細菌をもっています。症状が出るのが早いこと（早いときは1時間以内）が特徴です。局所の傷口が赤く腫れ，痛みや発熱を伴い，近くのリンパ節が腫れることもあります。また，かまれた部分の皮下に特徴的な，膿をもつ炎症（蜂窩織炎）をおこします（図2）。傷が関節に近かったり，深かったりすると，関節炎や骨髄炎をおこすこともあります。局所症状が主ですが，まれに敗血症など全身症状を示すこともあります。犬に引っかかれたり，なめられたりすることで感染することもあります。猫も保菌して感染源となります。ペニシリン系の抗生物質の早期の投与が有効です。

カプノサイトファーガ症：パスツレラ属菌と同様，犬の口腔内常在菌であるカプノサイトファーガ・カニモルサスなどカプノサイトファーガ属菌による感染症です。私たちの調査では95%程度，すなわちほとんどの犬がこの菌をもっていました。潜伏期は2～14日で，局所症状よりも全身症状が現れます。世界的にも患者報告数は非常に少ないのですが，敗血症や心内膜炎を発症したときの死亡率は約30%といわれています。髄膜炎を発症する場合もありますが，こちらの方が予後はよいようです。菌の増殖が遅いことや臨床現場での認知度が低いことから，気がつかない症例もあると考えられます。猫も感染源となります。パスツレラ属菌と同様にペニシリン系の抗生物質が有効です。

狂犬病：世界中で毎年5万人以上もの人が狂犬病で亡くなっています。世界保健機関（WHO）の報告では，感染源動物の99%が犬となっています。2006年の11月，1970年以来となる狂犬病患者が2例立て続けに報告されました。このような事

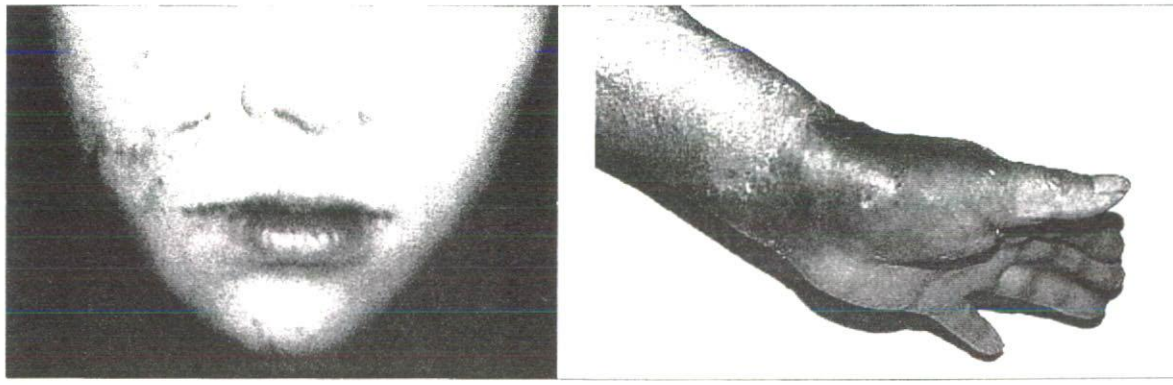


図2 パスツレラ症
左：犬による顔面の咬傷と炎症・腫脹。右：猫による手首咬傷部の腫脹。受傷後1か月。
（写真：日本大学医学部 荒島康友先生提供）

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*常在菌…体の決まった場所に日常的にいる菌で，普段は病気を起こさない。
*潜伏期…感染してから発症するまでの期間。

件があると，犬にかまれることが非常に心配になります。しかし，1970年の患者を含めてこれらの患者はいずれも海外で狂犬病の犬にかまれ，国内で発症したものです。1956年の人と犬，1957年の猫を最後に，国内での感染はありません。現在，日本は世界でも数少ない狂犬病清浄国なのです。ですから，国内の犬にかまれても今のところ狂犬病の心配はまずありません。ただ，近隣諸国では狂犬病が発生しているため，不法上陸犬*にかまれた人は，感染を想定して狂犬病ワクチン接種を受けることが望ましいとされます。WHOは，狂犬病の流行を防ぐには，全体の70%以上の犬にワクチン接種が必要だと述べています。日本では狂犬病予防法により，飼い犬の登録と狂犬病ワクチンを受けさせることが，飼い主に義務付けられています。

その他：傷口の膿から分離されてくることが多いものに，人の皮膚などにも常在している連鎖球菌やブドウ球菌などがあります。これらの菌も，まれに敗血症の原因となります。

破傷風菌はもともと土壌にいる菌なので，土で鼻や口，体を汚したりすることで犬に破傷風菌が付着することがあり，かまれたときに傷口から感染する可能性があります。現在，ほとんどの子どもはDPT混合ワクチン（DPT：ジフテリア，百日咳，破傷風）を接種していますが，破傷風ワクチンが混合ワクチンに加えられたのは1968年ですから，それ以前に生まれた人の中には接種していない人もいます。咬傷が原因で感染した例が多いわけではありませんが，転んで傷を作ったり，汚れたくぎなどで傷つけたり，汚染している土をいじったりすることなどで感染することがありますので，ワクチン未接種者や最後にワクチンを接種してから10年以上経過している人にはワクチン接種が望まれます。

感染・発症する危険性が高くなる要因は？

感染・発症の危険性はかまれた人の健康状態，咬傷部位や処置で変わってきます。

かまれた人：一般に免疫機能が低下していると危険性が高くなります。

①高齢者。
②アルコール依存症。
③糖尿病，肝硬変，脾臓の摘出を受けた。
④慢性関節リウマチなど自己免疫疾患。
⑤ステロイドホルモン（免疫機能を低下させる）の投与を受けている。

などは特に注意が必要です。

咬傷部位：傷口の状態によっても危険性が高くなります。

①腱，靱帯，関節にまで届くほどの深い傷を受けた（流水での洗浄や消毒が十分にできないことがある）。
②傷を受けた部分が汚れていた，もしくは汚れている。
③きちんと処置をしないまま傷口を放置した。
④きちんと処置をしないまま傷口を縫合した（内部に病原体が残ってしまう。空気に触れない方が増える病原体もある）。

などです。

かまれたときには，まずどうしよう？

傷の程度などによっても異なりますが，

①できる限り傷の奥の方まで，流水でよく洗う。ただし，無理に血液を絞り出そうとして患部をもんだりしない。誤って菌を傷の奥の方に押しやったり拡散させたりする元になる。
②異物（歯のかけら，毛，ゴミなど）や傷ついて取れそうな皮膚などが傷口にあれば取り除く。
③消毒（アルコールや市販の消毒薬などを利用）して，通気性のよい，清潔なガーゼなどを当てておく（可能ならば傷口を閉じない）。
④傷が大きい・深いとき，感染の危険性が高いときは医療機関を受診する。

というような対処をします。

当初は医療機関を受診していなくても，その後，症状が現れたり，体調に異変を感じたりしたときは，すぐに医療機関を受診し，医師に犬にかまれたことを伝えます。

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*不法上陸犬…検疫や正規の輸入手続きを受けず海外からやってきた犬。

また，咬傷事故は最寄りの保健所などに届け出ることになっています。かんだ犬の飼い主は，獣医師にその犬を受診させ，狂犬病かどうかの鑑定を受けさせなければなりません。

予防のためには？ 犬との接し方は？

①飼い犬のしつけをきちんとする（加害者にも被害者にもならないために）。
②自分の飼い犬が誰に対してもいい犬とは限らない，誰もが犬好きとは限らないことを理解しておく。
③知らない犬，様子のおかしい犬には近づかない，手を出さない。
④乳幼児だけを犬と一緒にしない（必ず大人がそばに付き添う）。
⑤犬を驚かせるようなことや，いやがることをしない。
⑥犬のじゃまをしない（えさを食べている，寝ている，子犬の世話をしている）。

など，犬を飼ううえでの基本的なマナーや，その習性を理解したうえでの接し方を考える必要があります。

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A latex agglutination test for the detection of *Echinococcus multilocularis* coproantigen in the definitive hosts

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Abstract

A latex agglutination test for detecting *Echinococcus multilocularis* coproantigen in definitive hosts was developed using latex beads sensitized with EmA9 monoclonal antibody raised against somatic antigens of adult *E. multilocularis*. A primary test (LA 1) was performed on 82 fecal samples of necropsied foxes, of which 46 were infected, and resulted in 61% sensitivity and 86% specificity. To increase the sensitivity, 4 ng/mL of excretory/secretory antigens of adult worms was added to the samples in a secondary test (LA 2), resulting in 91% sensitivity and 61% specificity. The positive predictive value of the LA 1 test and the negative predictive value of the LA 2 test were both 85%. The combination of the LA 1 and LA 2 tests is applicable and practical for use in situations that require quick diagnosis or screening based on the following interpretation: the samples that are positive in the LA 1 test are positive; the samples that are negative in the LA 2 test are negative; and the samples that are negative in the LA 1 test and positive in the LA 2 test are classified as suspicious.

Keywords: *Echinococcus multilocularis*; Latex agglutination test; ELISA; Coproantigen; Diagnosis; Definitive host

1. Introduction

Echinococcus multilocularis, one of the most serious zoonotic parasites, is widely distributed in the northern hemisphere, including Hokkaido, Japan (Eckert et al., 2001). Humans are infected by ingesting eggs derived from the feces of definitive hosts. In Hokkaido, the prevalence of *E. multilocularis* in red foxes, the main definitive host, has been approximately 40% over the last two decades. Moreover, infections of domestic dogs, which are another potential infectious source for humans because of their close proximity, have been reported not only on Hokkaido, but also on the main island of Japan (Kamiya et al., 2007; Morishima et al., 2006; Nonaka et al., 2006). In response to the deteriorating situation, a reporting system for canine echinococcosis has been enforced in Japan since October 2004; as of March 2007, seven cases have been detected.

The ability to perform a rapid and on-site diagnosis/screening for infection in definitive hosts would be beneficial for small animal practitioners in their risk management; however, this is difficult to perform with the currently available diagnostic tools. Fecal egg examination is inaccurate for *Echinococcus* species because of the morphological similarity of eggs among taeniid species and the intermittent excretion of eggs even after maturity (Eckert and Deplazes, 2001;

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