NOTE *Immunology*

Molecular Cloning and Sequencing of the cDNAs Encoding the Bat Interleukin (IL)-2, IL-4, IL-6, IL-10, IL-12p40, and Tumor Necrosis Factor-Alpha

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ABSTRACT. This is the first report on the cDNA sequences of bat interleukin (IL)-2, IL-4, IL-6, IL-10, IL-12 p40, and tumor necrosis factor (TNF)-α. The cDNAs of bat IL-2, IL-4, IL-6, IL-10, IL-12 p40, and TNF-α comprise 459, 405, 624, 537, 990, and 699 base pairs respectively. Moreover, each of the cDNAs of bat IL-2, IL-4, IL-6, IL-10, IL-12 p40, and TNF-α contain a single open reading frames encoding 152, 134, 207, 178, 329, and 232 amino acids, respectively. The comparison of bat cytokines with Perrissodactyla (horse), Carnivora (dog and cat), and Cetartiodactyla (cattle and pig) orthologs revealed a high degree of homology. Although the N-terminal amino acids and cysteine residues are highly conserved in each mature cytokine, the deduced N-linked glycosylation sites vary across species.
KEY WORDS: chiroptera, cytokine, molecular cloning, natural reservoir, zoonoses.

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Recently, there has been an increase in the incidence of infectious diseases originating from bats, such as rabies, Ebola virus, Nipah virus, and most likely severe acute respiratory syndrome (SARS) coronavirus [1, 14, 21]. In addition, several studies involving viral inoculations in bats suggest that some bat-associated pathogens cause less clinicopathological damage in this species, or have no effect, compared to other species [4, 10, 16, 19]. These findings imply that the cell-mediated immune responses of bats to viruses agents are different from those of other species. Although studies on fruit bats have revealed well-developed immune systems [15], lower levels of agglutinating, hemagglutinating, and complement-fixing antibodies are produced in response to various antigens in fruit bats than in conventional laboratory animals [5]. Furtherand, the peak of primary antibody response after antigenic challenge is delayed in fruit bats [2]. Additionally, the activation of T lymphocytes is significantly delayed in bats as compared to in mice [2, 3]. In the present study, we analyzed the cDNA sequences of bat Th1 (IL-2, and IL-12 p40) cytokines, Th2 (IL-4, IL-6, and IL-10) cytokines, and tumor necrosis factor (TNF)-α that was selected as the representative inflammatory cytokines. These cytokines have major roles in inflammation, and both cellular and humoral immunity. Th1 cells have a key role in the cellular immune response to viruses and other intracellular pathogens as well as in the elimination of cancer cells and stimulation of delayed-type hypersensitivity (DTH). On the other hand, Th2 cells drive humoral immunity and upregulate antibody production in order to neutralize extracellular organisms [8].

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Leschenault's Rousette bats (*Rousettus leschenaulti*) were maintained under controlled conditions using an air conditioner and moisture chamber. The animals were kept in steel cages, and fed fruit and water at the same time everyday. All experiments were performed in accordance with the Animal Experimentation Guidelines of the University of Tokyo, and were approved by the Institutional Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, The University of Tokyo.

Fresh spleen samples were collected from Leschenault's Rousette bats (*Rousettus leschenaulti*) under anesthesia with diethylether. The spleen was preserved in RNAlater (Ambion, Austin, TX, U.S.A.) and frozen at –80°C. Total RNA was isolated from bat spleen with TRIzol Reagent (Invitrogen, Carlsbad, CA, U.S.A.), according to the manufacturer's protocol. Primary cDNAs were synthesized by reverse transcription with Superscript®III reverse transcriptase (Invitrogen) using an Oligo $(dT)_{12-18}$ primer (Invitrogen), followed by PCR with Thermo-Start® Taq DNA Polymerase (ABgene, Epson, UK). To obtain cDNA clones of the middle region of the mRNAs of bat cytokines, reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using primer sets that are designed from the ortholog sequence data of horse, pig, and cat available on the GenBank database. The primers used for amplifying DNA fragments are described in Table 1. The amplified products were cloned into plasmids using a TOPO TA Cloning® Kit (Invitrogen), and to validate the sequences, 5 clones obtained for each sample were confirmed by DNA sequencing analysis using the ABI 3130/3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.). To determine the remaining 5'- and 3'-terminal gene sequences, both 5'-rapid amplification of cDNA ends (RACE) and 3'-RACE were performed using a 5'-full RACE core set (TaKaRa, Tokyo, Japan). To confirm the decoded bat inter-

Table 1. Primers used for amplifying the DNA fragments of each cytokines

Primer name	Sequence(5'–3')
TNF-α F1	AAGCATGATCCGAGATGTGGAGCTGGC
TNF-α R1	CTTCTCCAGCTGGAAGACTCCTCCCAGGTA
IL-2 F1	CTTGCACTTGTCGCAAACAGTGCACCTA
IL-2 R1	GCTTTGAGGTAAACCTAGCACTTCCTC
IL-4 F1	ACTAGCATGTACCAGCAACT
IL-4 R1	TTGGCTTCATTCACAGTACAGC
IL-6 F1	GGATGCTTCCAATCTGGGTTCAATCAGG
IL-6 R1	TGCCCAGTGGACAGGTTTCTGACCAGA
IL-10 F1	CCACATGCTCCATGAGCTCCGAG
IL-10 R1	GATGAAGATGTCAAACTCACTCATGGC
IL-12p40 F1	GGCTCTGGTAAAACTCTGACCATCCAAGTC
IL-12p40 R1	GAACCTAACTGCAGGACACAGATGCCCA

leukin nucleotide sequences, additional specific primers were designed on the basis of the first decoded sequences, and direct sequencing was carried out. The gene-specific primers are described in Table 2. KOD FX DNA polymerase (Toyobo, Osaka, Japan) was used for high-fidelity PCR amplification. The complete nucleotide sequences encoding bat IL-2, IL-4, IL-6, IL-10, IL-12 p40, and TNF-α comprised 459, 405, 624, 537, 990, and 699 base pairs, respectively, and the deduced amino acid sequences comprisedmade 152, 134, 207, 178, 329, and 232 amino acid residues, respectively. The cDNA sequences of IL-2, IL-4, IL-6, IL-10, IL-12 p40, and TNF-α were deposited in the GenBank database (Accession numbers: AB472358, AB472359, AB472360, AB472361, AB472362, and AB472363). The nucleotide and amino acid sequences were analyzed using the Genetyx-Win software (version 4.0; Software Development Co., Tokyo, Japan). The SignalP program (http://www.expasy.org) was used to predict the position of the N-terminal amino acids of mature proteins. The accession numbers of nucleotide data that were used for homology analysis are described in Table 3.

The sequence homology of the nucleotide and amino acid sequences of bat cytokines with other mammalian cytokine orthologs is described in Table 4. The alignment of the deduced amino acid sequences of bat, horse, dog, cat, cattle, pig, and human cytokines is described in Figs. 1A-1F. We found 4 conserved cysteine residues (positions 29, 31, 144, and 176) in bat TNF-α. In the TNF-α amino acid sequence, we found 6 amino acid residues (indicated by a rhomboid) involved in interactions at the TNF-α receptor binding site [7, 9]. Of these 6 amino acids in the bat, the Asp^{164} residue differed from that in other species. More research is required to determine whether this difference affects species-specific affinity of TNF-α for its receptor. In the bat, IL-2 contains 4 conserved cysteine residues (positions 9, 77, 124, and 144) and 1 potential N-linked glycosylation site (positions 109–111). Although the overall amino acid

Table 2. Primers used for confirming the decoded sequences

Primer name	Length of PCR product(bp)	Sequence(5'–3')
TNF-α F	898	CAGACAACACAGACCCGGAGAAGCA
TNF-α R		CTAATTCCCTTTCTGAAGAGGATGAG
IL-2 F	569	AGTATAAATTGCTCCTCTTGTTTG
IL-2 R		TAGCCTGATACATTTTAAGTGGGAG
IL-4 F	419	GATCTATTAATGGGTCTCACCTC
IL-4 R		CAGCTTCAACTCTTTGAGTATTTCTC
IL-6 F	762	TGCCCTCGAGCCCACCAGGAACGAA
IL-6 R		CATAAGTTATGTGCCCAGTGGACAG
IL-10 F	659	ACATCAGGGGCTTGCTCTTGCTCGACC
IL-10 R		TGTCCCAGGGTCTAGTAGAGTCGCCA
IL-12p40 F	1118	GTTTCACACCCAGAAAACTGC
IL-12p40 R		ATATCTTTTCCGGGTCGATTAG

Table 3. GenBank Accession numbers used in the sequence comparison

Species	GenBank Accession Numbers					
	TNF-α	IL-2	IL-4	IL-6	IL-10	IL-12p40
Horse	NM_001081819	NM_001085433	NM_001082519	NM_001082496	NM_001082490	NM_001082516
Dog	EU_249361	AM_238655	AF_239917	NM_001003301	NM_001003077	NM_001003292
Cat	NM_001009835	NM_001043337	NM_001043339	NM_001009211	AF060520	NM_001077413
Cattle	EU_276079	NM_180997	NM_173921	NM_173923	NM_174088	NM_174356
Pig	NM_214022	NM_213861	NM_214123	NM_214399	NM_214041	NM_214013
Human	NM_000594	NM_000586	NM_172348	NM_000600	NM_000572	NM_002187

Table 4. Sequence identities between the horse, dog, cat, cattle, pig, and human cytokines

Cytokine	%Nucleotide(%Amino acid) identity					
	Horse	Dog	Cat	Cattle	Pig	Human
TNF-α	88.7(85.9)	88.1(88.8)	88.3(88.4)	83.2(78.1)	85.2(85.8)	88.6(86.7)
IL-2	79.3(65.8)	78.6(67.5)	82.1(71.2)	75.2(57.8)	80.9(68.0)	80.7(65.8)
IL-12p40	91.2(90.2)	89.4(87.2)	88.6(86.6)	89.1(88.1)	88.3(86.3)	89.1(86.9)
IL-4	76.0(60.6)	74.6(61.8)	76.0(63.9)	76.6(68.1)	80.2(67.7)	68.0(45.9)
IL-6	79.4(67.1)	77.1(62.6)	77.5(55.7)	73.7(55.5)	79.8(66.5)	76.2(60.8)
IL-10	92.1(89.3)	87.7(86.1)	88.6(84.1)	86.1(80.9)	84.1(79.2)	89.1(83.7)

sequence homology was not high, the cysteine residues in IL-2 were highly conserved in the bat and other mammalian species. On the other hand, the N-linked glycosyilation site, which modulates the function of the mature IL-2 protein, was found to be conserved in the bat when compared to that in the horse, dog, cat, and pig; however, N-linked glycosylation sites in the putative IL-2 in cattle and humans are not found at the same sites as in the bat. After post-translational processing, Ala^{21} was deduced to be the first amino acid residue in the mature IL-2 protein at the N-terminal region. In the bat, IL-12 p40 contained 9 conserved cysteine residues (at positions 50, 90, 131, 142, 170, 194, 200, 301, and 328) and 3 predicted N-linked glycosylation sites (at positions 125–127, 135–137, and 223–225). Consistent with the results of a previous study, we found that the cysteine residues were entirely conserved among species [13]. Ile^{23} is at the putative N-terminus of the mature protein. Bat IL-4 has 5 conserved cysteine residues (at positions 13, 17, 48, 70, and 106) and 2 potential N-linked glycosylation sites (at positions 20–22 and 97–99). Although cysteine residues in IL-4 are highly conserved across species, another study [12] has reported that other mammals have an additional conserved cysteine residue at position 134 (indicated by a double circle in Fig. 1D). For IL-4, Arg^{25} is at the putative N-terminus of the mature protein. Bat IL-6 has 4 conserved cysteine residues (at positions 70, 76, 99, and 109) and 2 potential N-linked glycosylation sites (at positions 49–51 and 167–169). Although the cysteine residues were entirely conserved, the N-linked glycosylation sites in IL-6 may or may not be present depending on species. For IL-6, Pro^{27} is at the putative N-terminus of the mature protein. Bat IL-10 has 6 conserved cysteine residues (at positions 8, 9, 30, 80, 126, and 132) and 3 predicted N-linked glycosylation sites (positions 67–69, 100–102, and 134–136). A very high degree of sequence homology was observed in the amino acid sequence of bat IL-10 and IL-10 of other species, and the positions of cysteine residues were highly conserved in IL-10. However, the presence of additional N-linked glycosylation sites (Asn^{100}-Ser^{102}), which are absent in other mammalian species, may affect the function of bat IL-10. For IL-10, Ser^{19} is at the putative N-terminus of the mature protein.

In this study, the cDNA sequences of bat cytokines were analyzed. Overall, the comparison of bat cytokines with Perrissodactyla (horse), Carnivora (dog and cat), and Cetartiodactyla (cattle and pig) orthologs revealed a higher degree of homology in terms of the nucleotide and deduced amino acid sequences (Table 4). This is consistent with previous reports that conducted retroposon insertion analysis for examining phylogenetic relationships between organisms of almost all mammalian orders [11]. In addition, cysteine residues and N-terminus amino acids of mature cytokines are highly conserved among species [6, 17, 18, 20, 22]. However, N-linked glycosylation sites vary to some extent inposition and number. These results suggest that although the 3-D conformation of each cytokine is conserved among species, the glycosylation-related functions of each cytokine differ among species. Further research on different species-specific functions of each cytokine is needed to understand the characterization of immune systems in each animal.

The nucleotide sequence data obtained in this study will be very useful for future studies on the bat immune system. In particular, these data coupled with real-time RT-PCR for the quantification of the mRNA of bat cytokines will facilitate kinetic analysis of the cytokines. This is likely to be an excellent method for analysis of the immune system in wild animals, because a specific enzyme-linked immunosorbent assay (ELISA) systems for cytokine analysis across species is unavailable, and such a system would most likely take a long time to develop.

Further research on the bat immune system and infectious diseases originating from bats may provide key insights as to how bats act as natural reservoirs of infectious diseases. Further studies are needed to characterize the bat immune system and to understand the interactions of viruses and other pathogenic microbes with the bat immune system.

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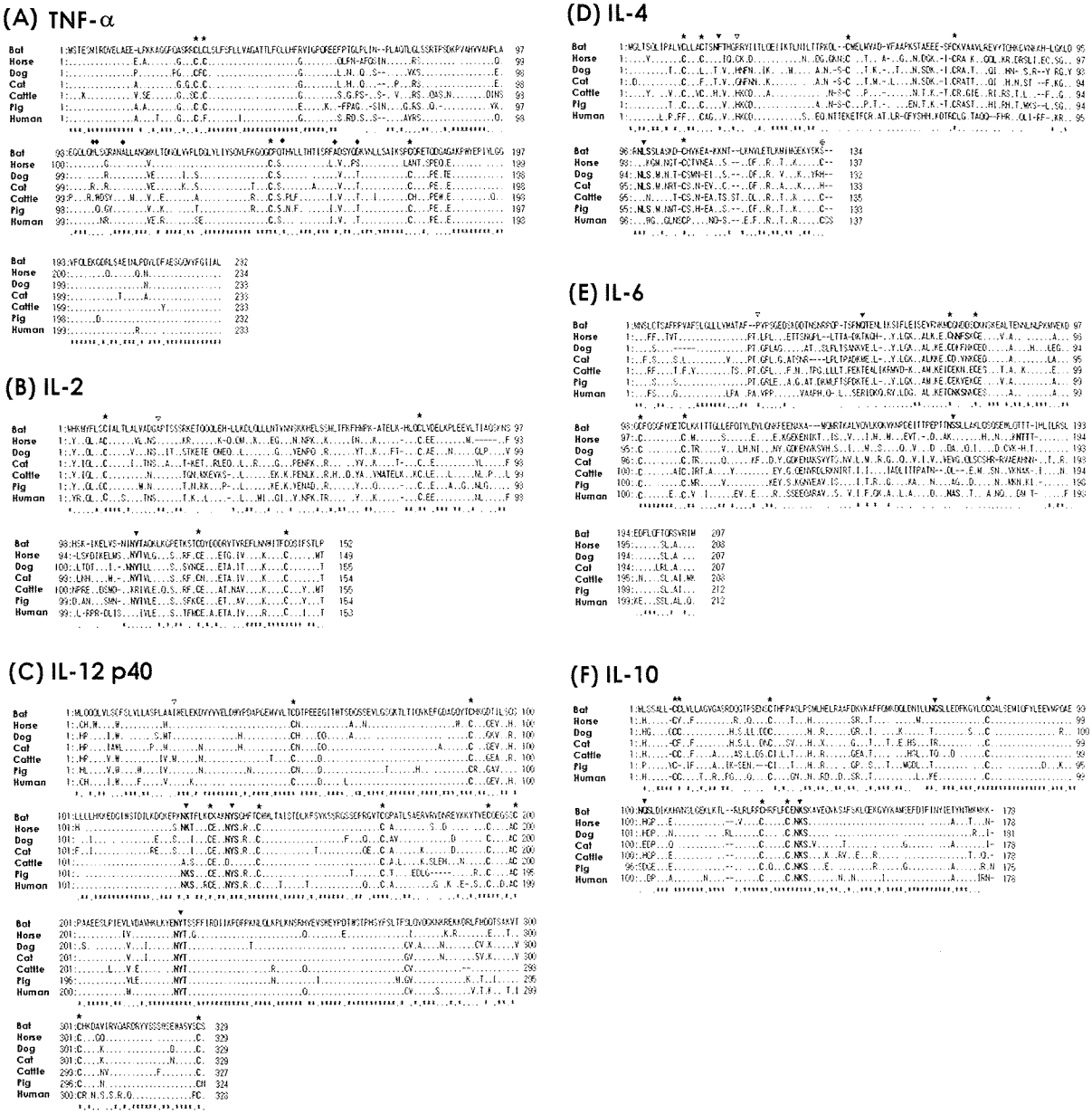

Fig. 1. Alignment of the deduced amino acid sequences of bat, horse, dog, cat, cattle, pig, and human cytokines ([A] TNF-α, [B] IL-2, [C] IL-12p40, [D] IL-4, [E] IL-6, and [F] IL-10). Identical amino acid residues are indicated by an asterisk (*) and almost identical amino acid residues are indicated by a dot (.). A black star indicates conserved cysteine residues, and a black arrowhead indicates a potential N-linked glycosylation site. White arrowheads indicate the amino acid terminus of the mature proteins. Amino acids involved in binding TNF-α receptors are indicated by a rhomboid. In Fig. 1D (IL-4), a double circle indicates a predicted substitution of a cysteine residue.

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Virus Genes
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Detection of a new bat gammaherpesvirus in the Philippines

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Abstract A new bat herpesvirus was detected in the spleen of an insectivorous bat (*Hipposideros diadema*, family *Hipposideridae*) collected on Panay Island, the Philippines. PCR analyses were performed using COnsensus-DEgenerate Hybrid Oligonucleotide Primers (CODEHOPs) targeting the herpesvirus DNA polymerase (DPOL) gene. Although we obtained PCR products with CODEHOPs, direct sequencing using the primers was not possible because of high degree of degeneracy. Direct sequencing technology developed in our rapid determination system of viral RNA sequences (RDV) was applied in this study, and a partial DPOL nucleotide sequence was determined. In addition, a partial gB gene nucleotide sequence was also determined using the same strategy. We connected the partial gB and DPOL sequences with long-distance PCR, and a 3741-bp nucleotide fragment, including the 3′ part of the gB gene and the 5′ part of the DPOL gene, was finally determined. Phylogenetic analysis showed that the sequence was novel and most similar to those of the subfamily *Gammaherpesvirinae*.

Keywords Bat virus · Direct sequencing · Herpesvirus · Virus discovery · RDV

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With the emergence of zoonotic viruses, including Nipah, Hendra, and Ebola viruses as well as severe acute respiratory syndrome (SARS) coronaviruses, there has been increasing interest in the role of bats as hosts for pathogens. Over 80 viruses have been isolated or detected in bats by nucleic acid analysis [1]. Herpesviruses are widely disseminated in vertebrates, and most mammalian orders have yielded at least one herpesvirus. However, no herpesviruses from bats are listed in the universal virus database [2]. A few herpesviruses were recently molecular characterized in bats [3, 4]. In this study, we examined bats in the Philippines in an attempt to find a new herpesvirus.

Forty-five insectivorous bats, *Miniopterus australis*, family *Vespertilionidae* ($n = 23$), *Miniopterus schreibersii*, family *Vespertilionidae* (17), *Miniopterus tristis*, family *Vespertilionidae* (2), *Rhinolophus arcuatus*, family *Rhinolophidae* (1), *Hipposideros diadema*, family *Hipposideridae* (1), *Myotis macrotarsus*, family *Vespertilionidae* (1), and one frugivorous bat, *Ptenochirus jagori*, family *Pteropodidae*

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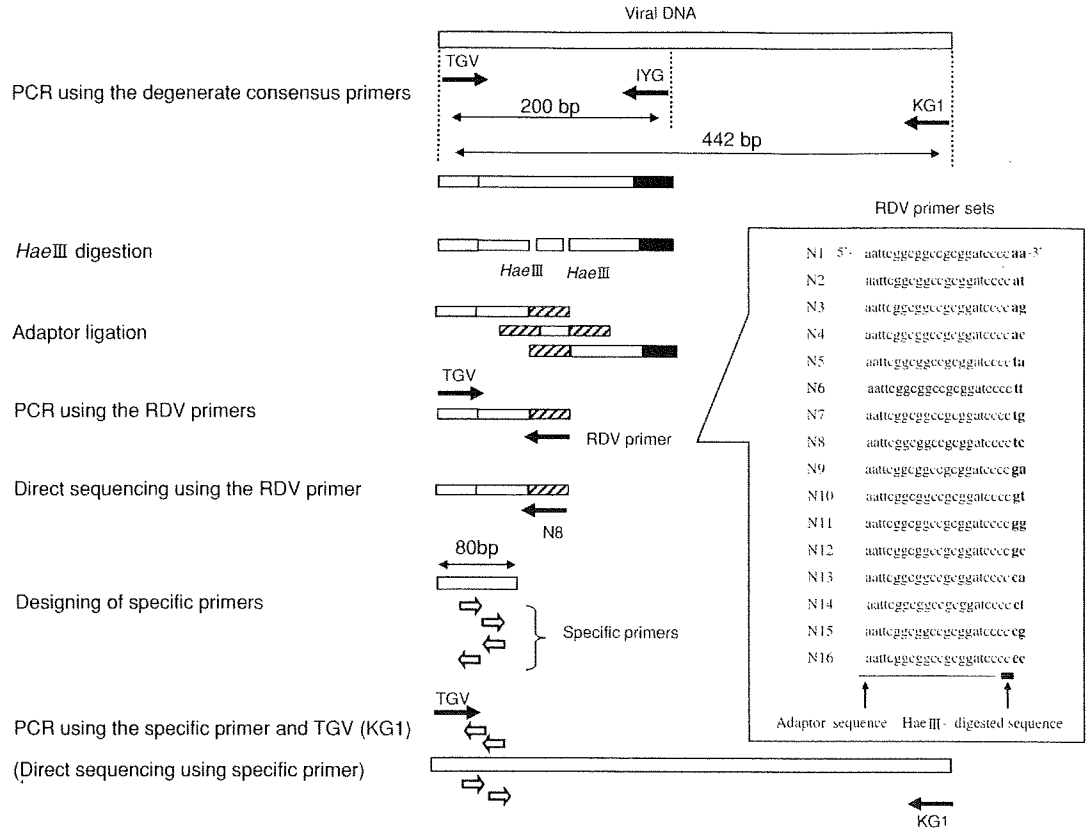

Fig. 1 Overall scheme for direct sequencing with RDV primer sets

(1), were collected at two sites on Panay Island, the Philippines, in 2008. The bats were euthanized under sedation as described previously [5]. Samples of approximately 100 mg of each spleen were used for DNA extraction using a QIAamp DNA mini kit (QIAGEN), according to the manufacturer's instructions. To detect herpesviruses in field samples, we used COnsensus-DEgenerate Hybrid Oligonucleotide Primers (CODEHOPs; nested PCR) for amplification of a partial herpesvirus DNA polymerase (DPOL) gene sequence [6]. These consensus primers are known to be effective for detecting herpesviruses from any vertebrate host. An amplicon of approximately 200 bp of the DPOL gene was obtained in the DNA sample from an insectivorous bat, *H. diadema* (data not shown). Although direct sequencing was performed using CODEHOPs to avoid contamination of DNA in our laboratory, this was difficult due to the high degree of degeneracy of the primers.

Recently, we developed a new method, rapid determination system of viral RNA sequences (RDV), for sequence-independent determination of viral fragment sequences without cloning [7–9]. As described in our previous reports and shown in Fig. 1, the RDV method includes direct sequencing technology. Each RDV primer contains the adaptor sequence, 4 nucleotides including CC (the end of the sequence after *Hae*III digestion) and 2 variable nucleotides. After purification of the 200-bp PCR product from the gel, DNA was digested with *Hae*III, and subjected to adaptor ligation as described previously [9]. PCR was performed using the RDV N1 to N16 primers and the degenerate TGV or IYG primer. We expected selective amplification of the templates having the RDV primer sequence and TGV (or IYG) primer at each end. PCR products were electrophoresed on agarose gels, and a DNA band of approximately 80 bp was obtained when the TGV and RDV N8 primers were used for amplification (data not shown). The DNA fragment was extracted from the gel, and direct sequencing was performed using the RDV N8 primer. Based on the fragment sequence obtained by direct sequencing, several specific primers were then newly designed. PCR was performed using these specific primers and the KG1 primer. The resultant PCR products were subjected to direct sequencing again. A 442-bp DPOL nucleotide fragment, corresponding to the region of amplification product with TGV and KG1, was determined (DDBJ accession no. AB459535).

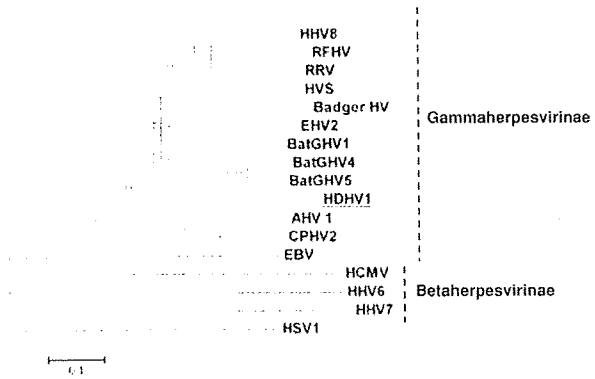

Fig. 2 A phylogenetic tree was constructed using a multiple alignment of 914 aa, consisting of concatenated gB and DPOL amino acid sequences. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. Phylogenetic analyses were conducted in MEGA4 [11]. The tree was rooted to herpes simplex virus type 1 (HSV1) (X14112). The evolutionary distances were computed using the Poisson correction method and are given in units of the number of amino acid substitutions per site. All positions containing alignment gaps and missing data were eliminated from the dataset. The herpesviruses used for comparison and their accession numbers are as follows: alcelaphine herpesvirus 1 (AHV1), NC_002531; badger herpesvirus (BadgerHV), AF376034; bat gammaherpesvirus 1 (BatGHV1), DQ788623; BatGHV4, DQ788627; BatGHV5, DQ788629; caprine herpesvirus 2 (CPHV2), AF283477; Epstein-Barr virus 1 (EBV)(human herpesvirus 4), NC_007605; equine herpesvirus 2 (EHV2), NC_001650; human cytomegalovirus (HCMV), NC_006273; human herpesvirus 6 (HHV6), AF157706; HHV7, NC_001716; HHV8 (Kaposi's sarcoma virus), NC_003409; retroperitoneal fibromatosis-associated herpesvirus (RFHV), AF005479; rhesus monkey rhadinovirus (RRV), AF083501; saimiriine herpesvirus 2 (HVS), NC_001350

BLAST search suggested that the DPOL sequence was novel and most similar to those of gammaherpesviruses. Therefore, PCR was performed with another nested primer set targeting the gB genes of gammaherpesviruses [4]. First PCR was performed using RH-gB 1s and RH-gB 1as primers. After second PCR with RH-gB 2s and RH-gB 2as primers, an amplicon of approximately 450 bp of gB gene was obtained. To determine the nucleotide sequence of partial gB genes using direct sequencing, the same strategy used for determination of the partial DPOL sequence was applied. As a result, a 631-bp gB nucleotide fragment, corresponding to the region of amplification product with RH-gB 1s and RH-gB 2as primers, was determined. Then, we connected the partial gB and DPOL sequences with long-distance PCR, using specific primers, which were designed based on the obtained sequences in gB and DPOL regions. A 3741-bp nucleotide fragment including the 3′ part of the gB gene and the 5′ part of the DPOL gene was finally determined and deposited in Genbank (DDBJ accession no. AB490083). In tblastn search, it was demonstrated that concatenated gB and DPOL deduced amino acid sequence (1146 aa) was novel and most similar to those of retroperitoneal fibromatosis-associated herpesvirus (58% amino acid sequence identity). We have tentatively named this virus "Hipposideros diadema herpesvirus 1 (HDHV1)." A phylogenetic tree was constructed using the neighbor-joining method with concatenated gB and DPOL deduced amino acid sequence (gB; 304 aa, DPOL; 610 aa) and the available sequences of known herpesviruses (Fig. 2). The tree confirmed that HDHV1 belongs to the *Gammaherpesvirinae* and suggested that HDHV1 is not assigned to the known genus. The tree also showed that HDHV1 is not placed in the same group with the known bat gammaherpesviruses. However, further characterization of HDHV1 is needed to reveal its taxonomic assignment.

Recently, bats have been described as hosts for herpesviruses in several countries in Europe, South America, and Asia [3, 4]. This report shows the detection of a new gammaherpesvirus in the Philippines, and confirms the wide geographical distribution of herpesviruses in bats. As bats display a high degree of diversity and account for 20% of the approximately 4,800 mammalian species [10], these animals are potential hosts for many unknown herpesviruses.

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Chapter 17

Current Status and Issues of Zoonotic Viral Diseases

Yasuhiro Yoshikawa

Opening comments

Human beings are heterotrophic organisms that depend on animals and plants as sources of nourishment. Most of our needs for protein and fat are now met by consumption of the milk, meat, internal organs and other parts of domestic animals. We have had a long relationship with domestic animals, some of which were already living among us when we started farming the land 10,000 years ago. A look at that history shows that almost all current infectious diseases suffered by humans have animal origins. In other words, diseases such as smallpox, measles, and influenza that were once thought to be unique to humans, all pathogens originate in other animals or share common ancestors with viruses infecting other animals. There are also many infectious diseases even today that can be passed between people and domestic animals. We humans do not inhabit a special world separate from that of other animals.

From animals to humans

Zoonotic diseases are diseases caused by a pathogen that infects both animals and humans (but natural hosts infected by the pathogen often do not suffer any adverse effects). They consist mostly of diseases passed on to humans from animals, and diseases originally passed on to animals from humans and then back to humans from the infected animals (so-called recurrent infections, e.g. dysentery, tuberculosis, viral hepatitis, and other diseases found in monkeys).

Zoonotic diseases include such well-known examples from ancient times as plague, which is transferred from wild rodents (rats, etc.) to humans through fleas (and is by no means a disease of the past, still being prevalent in the continents of Africa, Asia, and America), and rabies, which is passed on to humans from infected dogs, bats, and other animals. There are of course many other parasitical, rickettsial and chlamydial, bacterial, and viral diseases affecting humans. In 1959, a WHO and FAO joint expert committee listed over 150 such diseases, and now there are thought to be 500-700 noteworthy diseases. Infectious diseases that have sent shockwaves throughout the world in recent times include diseases of wild

animal origin such as Ebola hemorrhagic fever (HF), Nipah virus infection, SARS, and West Nile fever; diseases of domestic animal origin such as O-157, BSE, and HPAIV; and diseases of arthropod origin such as dengue fever, dengue hemorrhagic fever, and malaria. About two-thirds of all viral diseases to have emerged in the latter half of the 20th century are zoonotic. Infectious diseases of domestic animal origin such as salmonella, hepatitis E, O-157, and BSE warrant serious consideration also from the food safety perspective since they invariably spread through foodstuffs.

Retrospectively, it was in 1980 that the WHO declared that smallpox had been eradicated. Though it is only one pathogen, this was the first time in history that mankind had defeated a virus (though recently people have voiced concern that it has not been completely eradicated ironically insofar as it continues to exist in the form of samples that might some day be used as pathogens in acts of bioterrorism). With the development of antibiotics, we also became able to suppress bacterial infections, giving rise to optimism about our ability to protect ourselves from infectious diseases. In Japan too, the infectious diseases that were long the top causes of death declined rapidly after the 2nd World war, making way for cancer to become the No.1 cause of death by 1950. As circulatory disorders became the 2nd most prominent cause of death, Japan's healthcare authorities began to focus more on welfare and countering cancer and lifestyle diseases rather than infectious diseases.

However, new infectious diseases such as AIDS and various viral hemorrhagic fevers have emerged worldwide, and diseases such as dengue fever and tuberculosis have reemerged to become serious threats to human health once again. Excessive use of antibiotics has given rise to the spread within hospitals of antibiotic-resistant bacteria such as MRSA, VRE, and VRSA. Given such developments, the WHO has revised its optimistic forecasts regarding the fight against infectious diseases, and countries throughout the world have declared states of crisis with regard to infectious diseases.

Factors behind the occurrence and spread of zoonotic diseases

Most zoonotic diseases can be traced to developing countries. The reasons for this include increased contact with pathogens carried by wild animals in tropical rainforest and other natural habitats during development of human production activities (Ebola HF, Marburg disease, monkeypox), disturbance of ecosystems by rodents and other animals whose numbers have been elevated by increased human productivity (Bolivian HF, Lassa fever, Argentine HF, etc.), establishment of infectious disease in cities of developing countries, which is normally circulated between monkeys and mosquitoes in forests owing to rapid urbanization and population concentration combined with poor urban infrastructure (yellow fever, dengue fever, dengue HF, etc.), and rapid spread of infection from developing to developed countries as a result of the rapid air transport of both people and animals (Lassa fever, Marburg disease, SARS).

There are also contributing factors in developed countries, such as the keeping of wild animals as so-called exotic pets (tularemia, plague, monkeypox, etc. transmitted by pet prairie dogs), and contact with wild animals during outdoor recreation such as camping or

forest walking (Japanese spotted fever, scrub typhus, Hantavirus pulmonary syndrome and Lyme disease transmitted by such animals as wild rodents and ticks, echinococcosis transmitted by foxes, etc.). New infectious diseases have also emerged in developed countries as a result of the pursuit of economic efficiencies in the form of intensive factory farming and rendering of animal parts as sources of protein (salmonella, BSE, O-157, etc.). In recent years, moreover, we are seeing transmission patterns of a more complicated kind, such as the Hendra and Nipah viruses transmitted from tropical fruit bats — up to now not known to be carriers of pathogens — to humans through domestic animals.

The chances of coming into contact with infectious diseases in humans transmitted by domestic animals such as pigs (Nipah virus), horses (Hendra virus), cattle (BSE), or chickens (HPAIV) are much higher than for those of wild animal origin. Domestic animals are increasingly raised for human consumption in large-scale factory farms, and once a pathogen invades such an intensive rearing environment, it can spread like wildfire, with the likelihood that its frequent transmission among hosts in such an environment will also facilitate genetic mutation, making for a much more dangerous situation than in the past.

Even among wild animals, we might be facing new risks. For example, increasing environmental pollution might reduce host immune functions, as a result of which a virus that has up to now coexisted with a host suddenly begins to spread explosively (North Sea seal virus, etc.), or environmental pollutants might elevate the frequency of virus mutation, because they were frequently mutagenic chemical substances. This kind of possibility suggests a need for conception change and actions different from earlier measures for suppressing zoonoses and avoiding risks. Conservation medicine (http://en.....) is a new approach to the control of zoonoses that incorporates the concept of environmental conservation in the consideration human and animal health.

Warning to humanity

The way in which zoonoses emerge and spread is changing in connection with the expansion of human production activities, pursuit of economic efficiency, changing lifestyles, and so forth. In this respect, zoonoses have much in common with environmental pollutants such as PCB, DDT, and dioxins. There is nothing evil about pursuing comfort and convenience, but if in our anthropocentric pursuit of ever more advanced technology we continue to ignore the need for balance and continue to destroy the environment and ecosystem, we are doomed to suffer the consequences. Attempts to resolve issues by pushing the contradictions of developed countries onto developing countries or by a country just looking out for itself are already proving to be bankrupt. What is needed is global cooperation between governments on countermeasures to zoonoses led by the WHO and OIE. National governments should also be remind that to avoid covering up or failing to report outbreaks, or all clear declarations under issuing premature. Other acts aimed simply at protecting one's own country's economy or calming the populace will in the end only raise the risks of a global infection (SARS in China, HPAIV in Southeast Asia, BSE in the UK, etc.).

Even the USA, which has the most advanced infectious disease defense system in the

world and is home to the Centers for Disease Control and Prevention (CDC) that plays a leading role in controlling infectious diseases worldwide, has not had an easy time controlling zoonoses like West Nile fever that are transmitted through wild animals (birds and mosquitoes). West Nile fever first appeared in eastern New York in 1999, infecting 7 people, but by 2003, it had spread throughout the country and still shows no signs of abating, with infections now standing at over 8,000 and deaths at over 200. The USA is also finding it extremely difficult to suppress plague endemic to arid Midwest regions (being transmitted between prairie dogs and fleas) and rabies transmitted by bats.

Meanwhile, the fact that SARS, which is thought to be of wild animal origin, spread throughout the world in a matter of months demonstrates that national borders and other artificial barriers are no obstacle to modern infectious diseases. HPAIV H5N1, the subject of this symposium, has also spread from Asia to the Middle East, Europe, and Africa. The number of countries affected, the scale of infection, and virulence that has enabled it to directly infect not only pigs but also humans, has prompted the WHO to issue dire warnings about the dangers it poses. In addition to conventional downstream, end-result-oriented infection countermeasures targeting people and animals (Ministry of Agriculture, Forestry and Fisheries [MAFF], Ministry of Health, Labour and Welfare [MHLW]), in the 21st century, zoonoses originating in wild animals need to be investigated from a more upstream perspective that also considers the environment and the ecology of pathogens parasitizing wild animals and natural hosts in order to develop more global countermeasures.

The path to controlling zoonoses

Including pathogenic microorganisms, there are currently about 1.4 million known species on Earth (approximately 750,000 insects, 280,000 other animals, 250,000 higher plants, 70,000 fungi, 30,000 protozoans, 5,000 bacteria, and 1,000 viruses). When one considers the complexity of the ecosystem that these organisms have built up as the present-day descendants of 3.7 billion years of life on Earth, it is impossible for we humans to completely control zoonoses for the sake of our own convenience. Basically we need to recognize the importance of biodiversity and seek to achieve a balanced coexistence with other life forms.

Even so, we need to do what we can to control infectious diseases that endanger humanity. The organizations charged with the responsibility of controlling infectious diseases on an international level are the Geneva-based WHO for human infectious diseases, and the OIE, headquartered in France, for animal infectious diseases and infectious diseases whose origins can be traced to foodstuffs. Because OIE decisions frequently directly affect domestic animals in various countries and trade in foodstuffs of domestic animal origin, the OIE also serves as an affiliate of the WTO.

The expert committees of these international organizations frequently use risk analysis as an analytical method. This methodology was originally used to decide international safety criteria with respect to humans for drugs, food additives, and so forth, but has come to be used also in the control of food poisoning and infection by microorganisms. Risk analysis is a field that merges natural science with social science, and is made up of three key aspects –

risk assessment, risk management, and risk communication. Based on a scientific, quantitative risk assessment, the parties concerned (risk managers) consider cost-effectiveness and draft a realistic plan that they explain to others in easily understandable terms, and attempt to establish a more efficient defense system. In Japan after the BSE panic, the Food Safety Commission was established within the Cabinet Office as a risk assessment organ independent from risk management organs. International organizations are already bringing together infectious disease experts and government officials from different countries or regions in field-specific forums to consider measures for the sustained control of infectious diseases.

However, the control of such diseases is basically a political and economic issue. As long as poverty, famine, and war continue, there is little hope for improving public hygiene globally. The path to controlling infectious diseases is one of international cooperation in the building of standards and systems for global defense against such diseases that also respect diversity in the form of national and regional differences in culture, national character, and everyday life and customs.

Japan's new zoonosis countermeasures

After the postwar period of rapid economic growth, dramatic changes in the social system and values fueled the trend towards nuclear families and declining birthrate, and pets as companion animals came to serve as substitutes for people. Then during the economic bubble of the 1980s, in place of the traditional species of pet animals, the import and keeping of exotic animals became very popular. Japan's birthrate declined and population aged at a pace that was exceptional even among the developed countries, and Japan also stood out from the rest in the quantity of its wild animal imports. These changes in society and diversification in lifestyles prompted increasing concern over the possibility that novel zoonoses would emerge, and so when the Infectious Diseases Control Law was enacted (effective from 1999), in addition to diseases transmitted between people, zoonoses too were considered for the first time, and with an expansion of the Rabies Prevention Law, cats, skunks, raccoons, and foxes in addition to dogs became subject to legal quarantine, as did monkeys. However, other infectious diseases and animal species were not subject to regulation, and so when the Infectious Diseases Control Law came up for revision 5 years later, stronger measures were considered.

For this revision, data on infectious diseases, the realities of imported animals, and disease risk assessment was obtained and analyzed. An MHLW zoonotic disease study team carried out a first-ever zoonosis risk analysis. As a result, a total import ban was imposed on all Chiroptera (bats) and rodents of the Mastomys genus (the natural hosts of Lassa fever) from November 2003, and requirements such as import notification, health certificates, and tethering according to risk level were applied to all other animals apart from prairie dogs and civet cats whose import was already prohibited, and monkeys and carnivores already subject to legal quarantine. In other words, unlike previous revisions which tended to simply increase animal quarantine, the new revision applied import bans to certain species, tethering orders, stronger measures against introduced animals and indigenous wildlife (migratory birds,

crows, etc.) including surveillance systems, investigation of animals in the event of outbreak of a zoonosis, and stronger measures to combat zoonoses. Particularly the animal import notification system and requirements for health certificates and furnishing of proof of non-infection with certain pathogens effectively put a stop to the import of wild animals that had gone unchecked up to then, and this has proved to be an effective alternative to quarantine as a means of avoiding risks.

With respect to wild and domestic animals within Japan, everyday surveillance is vital, which means that it is also vital to establish an organization for diagnosing infections in animals. With regard to high-risk infectious diseases, there is a need to identify high-risk localities, localities in which animal intrusion is likely, and habitats of wild animals carrying the infectious diseases concerned, and take comprehensive measures to combat the spread of the disease, curb the number and habitats of natural hosts and animal vectors, exterminate intruders, and so forth. This is a field that calls for cooperation between central and local government, between MAFF and the MHLW, and between doctors and veterinarians.

特集

人と動物の共通感染症最前線　6

動物病院勤務者の人獣共通感染症にかかわる健康調査

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要　約

人とペット動物間の共通感染症の実態を明らかにするために，動物病院勤務者の健康調査を行った。北九州市，神戸市，埼玉県西支部の323名から協力が得られた。抗体陽性率は猫ひっかき病11.2%，トキソプラスマ症4.3%，オウム病4.0%，犬ブルセラ症1.2%，Q熱0.7%であり，65名（20.1%）の者が抗体陽性を示した。ペット飼育者，動物病院勤務者の健康管理およびペット取扱衛生管理は十分に行われるべきものと思われた。

研究目的

国内におけるペットブームは今も続いており，家庭におけるペット飼育率は40%といわれている。また，多くのペットは伴侶動物として人々の生活に深く関わっているが，人とペット動物間の感染症伝播の実態は明らかになっていない。この状況下で，ペット動物由来感染症に関してハイリスクグループと考えられる動物病院勤務者の健康調査を行った。

対象および方法

北九州市，神戸市，埼玉県西支部の獣医師会の了承のもと，健康調査に協力すると意思表示をした動物病院勤務者を対象とした。この調査は東京大学農学部生命科学研究科倫理委員会の承認のもとに実施され，アンケート調査，血液抗体検査および結果統計処理が行われた。調査研究期間は平成18年1月から平成20年10月に及んだ。アンケートでは，年齢，性別，勤続年数，職種，動物との接触時間，取扱動物種，勤務者の衛生対策，感染経験の有無と対処方法，健康管理の有無，自宅でのペット飼育状況など11項目につき回答を求めた。抗体測定は8項目〔腎症候性出血熱（HFRS），リンパ球性脈絡髄膜炎（LCM），レプトスピラ症，猫ひっかき病，トキソプラスマ症，オウム病，犬ブルセラ症，Q熱〕を型通りの検査法にて実施した。

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結　果

①調査協力者は323名，男性114名（平均年齢42.1歳±12.0歳）女性209名（平均年齢30.0歳±9.2歳）であった。②職種分類は，獣医師135名，獣医看護師151名，トリマー12名，事務14名，行政関係者7名であった。③調査協力者の79.8%は今回の調査時に自宅でペット飼育中であり，過去・現在ともに自宅でペット飼育の経験が無い者はわずか3.2%であった。④抗体測定の結果は表1に示す。HFRS，LCM，レプトスピラ症の抗体陽性者は皆無であった。地域別，男女別比較では抗体陽性率に差は見られなかった。いずれか一項目が陽性であった者は65名(20.1%)，複数項目が陽性であった者は4名(1.2%)であった。⑤勤続年数につき検討すると，男性獣医師で11年以上の勤務者では抗体陽性率は有意（$p = 0.028$）に高率であった。⑥動物やケージ等を取り扱う時に，自分自身や動物を守るために実施している衛生処置としては，「手洗い」「爪切り」「使用器具の消毒」が主に実施されており（実施率96%，66%，74%），帽子をかぶる3%，手袋の着用26%，マスク着用13%，専用履物の使用34%，作業着の着用48%，うがいの実施5%。シャワー浴は2%

表1　抗体陽性率（項目別・地域別）

	埼玉（n=163）			神戸（n=90）			北九州（n=70）			総計（n=323）	陽性率（%）
	男性	女性	合計	男性	女性	合計	男性	女性	合計		
B. Henselae 抗体	6	10	16	4	8	12	1	7	8	36	11.15
Toxoplasma 抗体	8	0	8	1	1	2	0	4	4	14	4.33
オウム病	1	5	6	3	2	5	1	1	2	13	4.02
犬ブルセラ	0	1	1	1	1	2	0	1	1	4	1.24
Q熱*	0	0	0	1	0	1	1	0	1	2	0.66

* Q熱のみ19名（神戸：女性6名，北九州：男性2名，女性11名）のデータが欠損

いずれか1つ以上の抗体が陽性：65名（20.12%），うち複数の抗体が陽性：4名（全体の1.24%）

と低率であった。これら衛生処置の実施の有無と各種抗体価の陽性者との間には統計学的関連性は見いだせなかったが，「手袋の着用」「うがいの実施」「帽子着用」まで実施している動物病院勤務者の抗体陽性率は有意に低値であった。（$p = 0.46$）⑦診療動物による咬傷，ノミ刺傷の体験者は獣医師で126名(93.3%)，獣医看護師126名(91.4%)，トリマー11名（91.7%）とほとんどの勤務者が咬傷・刺傷の体験者であった。この体験時に病院を受診した者は，獣医師27.7%，獣医看護師37.7%，トリマー54.5%にとどまっていた。動物病院勤務者自身で自己診断治療を行ったことがある者は72.4%に及んでいた。されど，動物咬傷経験と抗体陽性者との間には統計学的に有意な関連性はみられなかった。⑧自己の健康管理に関して，健康診断・人間ドッグの受診率は31.0%にとどまっていた。

考　察

北九州市，神戸市，埼玉県西部の3地域において，323名の動物病院勤務者の健康調査が行えた。対象者数としてはやや少ないかもしれないが，九州地区，関西地区，関東地区での地域性の偏在も含め検討できたかと思われる。地域差の確認ができたのは，犬，猫以外のエキゾチックアニマルがいずれの地域でも飼育されてはいるが，動物病院での診療比率は埼玉西部，神戸市，北九州市の順に有意に高いものであった。10年前に吉川らの行ったエキゾチックアニマルの診療実態では，都市部と地方とには差が見られないとの結果であったが，現状では3地域での診療比率は埼玉西部で有意に高率で北九州市では低率であった。しかしながら，診療対象動物としての地域差，診療体制，衛生処置，動物咬傷・ノミ刺傷経験，自己の健康管理，動物との接触時間，自宅でのペット飼育には3地域における地域差は見られず，おおむね同一条件下での健康状態の比較検討ができたものと思われる。

この状況下，調査対象とした動物病院勤務者は患獣との接触リスクが高いグループと想定して血清抗体価陽性の有無との関連につき検討を行ったが，対象とした動物病院勤務者は自宅でのペット飼育率が79.8%以上と高率であり，動物病院での感染リスクばかりではないグループの健康調査になったかもしれない。このような人獣共通感染症のハイリスクグループにおける8項目に及ぶ血清抗体価測定をそれぞれの専門機関において行い，アンケートの回答とをあわせ統計学的解析を行った。その結果，猫ひっかき病，トキソプラスマ症，オウム病，犬ブルセラ症，Q熱の5項目で65名(20.1%)がいずれかの項目で陽性であった。

この15年間，これらの項目の疾病に関する個別症例報告はあっても，本報告のような疫学調査報告はなく，今回の調査結果のような抗体陽性率の高さをどの様に評価するかは難しいものがある。しかしながら，本研究における血清抗体価の測定は最も信頼できる研究機関での確実な検査法に基ずいた結果であり，それぞれ個別の抗体陽性率は表1に示すように納得できる値である。このことを踏まえれば，動物病院勤務者の抗体陽性率が20%は納得できるもののように思われる。また，抗体陽性率の地域差に有意差はみられず，国内全域において同様な傾向がみられるものと推測される。今後の疫学調査，健康管理の対策検討の参考に活用されることを願いたい。

さらに，動物病院勤務年数が長くなるほど抗体陽性率が高くなる傾向があり，特に勤続11年以上の男性獣医師の抗体陽性率は有意に高率であったこと，そして動物病院勤務者の多くが動物咬傷等の体験を持つにもかかわらず，自己診断・自己治療で事を済ませていることは由々しきことと思われる。状況を踏まえ，医療機関での診療が望まれるが，抗体検査はじめ，正確な検査法がルーチン検査として確立されることが望まれる。

2006年，我が国ではようやく輸入ペット動物の届出制

度が確立され，野生ペット動物の輸入には大きく制約がかけられたとはいえ，「人と動物の共通感染症」は決して減少せず，むしろ，これからの地球上での新たな発生が危惧されている。動物病院勤務者においても，自宅でのペット飼育者においても，衛生管理や動物との接し方に十分な注意を払うことは重要なことである。人もペット動物もともに健康でいられてこそ，意義のある生活が保たれるものと思われる。

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特集：野生動物の国際移動に伴う感染症拡大をいかに防ぐか
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International Wildlife-Disease Notification System

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ABSTRACT. New mission of the OIE on the international wildlife disease notification system was introduced. Main purpose of this WAHIS/Wild system is collecting and presenting the worldwide data of wildlife diseases by OIE to each member country using the WAHIS/Wild database. It is useful for control zoonoses, domestic animal infections derived from wildlife as well as maintain ecosystem and conservation of biodiversity. The basic tactics of the system which was discussed in the *ad hoc* group of the Working Group of Wildlife Diseases in OIE are summarized. Present situation and problems of wildlife disease notification in Japan were also described here. (This is a record of a keynote lecture of the 14th Japan Anual Congress of the Wildlife Medicine in Kobe, 2008)

Key words：wildlife, zoonosis, OIE-listed diseases, HPAI, Chytridiomycosis

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1. Introduction

Recent world public health threats are thought to be resulted from "an emerging and re-emerging diseases". These infectious diseases were mainly derived from pathogens of animals (zoonosis). Zoonosis outbreak around the world might be related to human activities both in developing and developed countries. For example, they are BSE (bovine spongiform encephalopathy) outbreak by the use of meat and bone meal (MBM) in UK and viral hemorrhagic fever outbreaks by rain-forest development in tropical countries in Africa. Conventional downstream, end-result-oriented infection countermeasures, taken by the Ministry of Agriculture or Ministry of Public Health, targeting people and domesticated animals, have been old fashioned. It is now needed to change a paradigm that investigations start from more upstream perspectives, i.e., to consider the environment, ecosystem and the ecology of pathogens parasitizing wild animals and natural hosts.

In addition, disease in wildlife may adversely affect not only human public health but also livestock health, as well as conservation of wildlife in native habitats, and hamper international translocation initiatives. However, data of international wildlife disease are comprehensively covered by neither World Health Organization (WHO) nor Food and Agriculture Organization (FAO), thus far. The OIE (Office de International Epizootics: now World Organization for Animal Health) is asked to conduct this work by negotiations with

Wildlife Disease Control Center (WDCC)

New strategy of OIE on wildlife diseases was opened (collaboration with FAO and WHO)

Disease control of wildlife becomes a new target of OIE
New notification system of wildlife diseases will be started
Statement of the Director General, Dr. Bernard Vallat was declared on OIE top page (2008.07.15)

Purpose: Establishment of notification system of wildlife diseases because of
1, zoonosis control for human public health (WHO, OIE)
2, diseases control of domestic animals for food security and food safety (FAO,OIE)
3, wildlife disease control for maintain ecosystem and conservation of biodiversity (OIE)

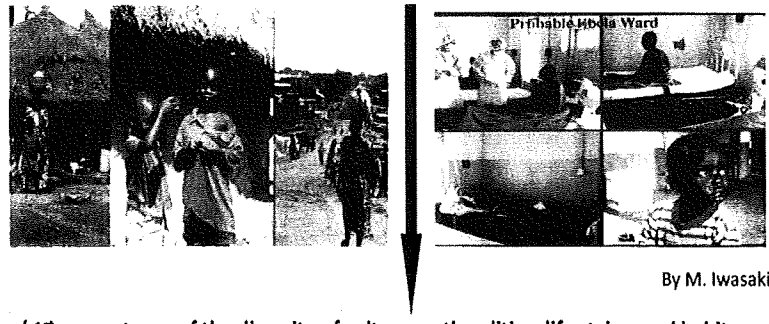
other two international organizations (WHO and FAO). Therefore, OIE makes efforts more actively not only from the stand points of food borne disease and zoonosis-control but from the stand points of wildlife conservation and ecosystem. At the same time, the OIE is sensitive to a potential for misinterpretation of information concerning wildlife disease occurrences and possible consequences to trade in animals and animal products associated with such misinterpretations. It is important how best to gather and report on occurrences of wild animal diseases without provoking unjustified trade restrictions.

2. Biodiversity and disease control in the world

Including pathogenic microorganisms, there are currently about 1.4 million known species which are living on the Earth (approximately 750,000 insects, 280,000 other animals, 250,000 higher plants, 70,000 fungi, 30,000 protozoa, 5,000 bacteria, and 1,000 viruses). When one considers the complexity of the ecosystems that these organisms have built up as the present-day descendants of 3.7 billion years of life on the Earth, it is impossible for us humans to completely control wildlife diseases for the sake of our own convenience. Basically we need to recognize the importance of biodiversity and seek to achieve a balanced coexistence with other life forms (segregation of our life milieu from territories of wildlife), such as compartmentalization or zoning between our lives and those of wildlife.

Even so, we need to do what we can to control infectious diseases that endanger humanity, domestic animals and wildlife. The organizations charged with responsibility for controlling infectious diseases on an international level are the Geneva-based WHO for human infectious diseases, and the OIE, headquartered Paris in France, for infectious diseases among domestic animals and infectious diseases whose origins can be traced to foodstuffs. OIE decisions directly affect the husbandry of domestic animals in all member countries (now 176 countries) and trade in foodstuffs of domestic animal origin, because the OIE serves as an affiliate of the WTO (World Trade Organization).

The expert committees of these international organizations frequently use "risk analysis" as an analytical method. This methodology was recently established and originally used to decide international safety criteria with respect to humans for drugs, food additives, and so forth, but has come to be used also in the control of food poisoning and infection by microorganisms. Risk analysis is a field that merges natural science with social science, and comprises three key aspects i.e., risk assessment, risk management, and risk communication. Based on scientific, quantitative or qualitative risk assessment, the risk managers or policy makers concerned consider cost-effectiveness, cost-benefit or trade-offs of the risk, etc., and draft a realistic plan that they explain to stakeholders in easily understandable terms (to perform accountability and to get informed consent), and attempt to establish a more



By M. Iwasaki

/ 1st, acceptance of the diversity of culture, nationalities, life styles, and habits in individual countries or areas is very important

/ 2nd, it is important to make a global standard and network system for zoonosis-control based on this acceptance, and international harmonization and collaboration are keys for developing a disease prevention road-map