humans and then back to humans from the infected animals are called anthropozoonoses (*e.g.*, dysentery, tuberculosis, viral hepatitis, and other diseases found in non-human primates).

Zoonotic diseases include such well-known examples from ancient times as the plague, which is transferred from wild rodents (rats, prairie dogs, etc.) to humans through fleas. It is by no means a disease of the past and nowadays the plague is still prevalent in the continents of Africa, Asia and America. Rabies, which is passed on to humans from infected dogs, bats and other animals, is reported to cause more than 40,000 fatalities a year, and several tens of millions of people are post-exposure vaccinated all over the world every year.

There are of course many other parasitic, rickettsial and chlamydial, bacterial and viral diseases affecting humans. In 1959, a World Health Organization (WHO) and Food and Agriculture Organization (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, severe acute respiratory syndrome caused by a corona virus (SARS), and West Nile fever; diseases of domestic animal origin such as enterohemorrhagic *E. coli* infection (EHEC) caused by O-157, bovine spongiform encephalopathy (BSE, a prion disease resulting in variant Creutzfeldt Jacob disease; vCJD), and highly pathogenic avian influenza (HPAIV); and diseases of arthropod origin such as dengue fever and dengue hemorrhagic fever. About two-thirds of all viral diseases emerging in the latter half of the 20th century are zoonotic (Table 1). Infectious diseases of domestic animal origin such as toxoplasmosis, salmonella and campylobacter infections, hepatitis E, O-157 enteritis, Crimean-Congo hemorrhagic fever, bovine tuberculosis, brucellosis, and BSE-vCJD also warrant serious consideration 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 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, infectious diseases that were long the top causes of death declined rapidly after the 2nd World War, making way for cancer to become the leading cause of death by 1951. As circulatory disorders such as brain stroke and myocardial infarction became the second most prominent cause of death, Japan's healthcare authorities (Ministry of Health Labor and Welfare; MHLW) began to focus more on welfare and countering cancer and lifestyle disorders, so called "metabolic syndrome," rather than infectious diseases.

However, new infectious diseases such as acquired immune deficiency syndrome (AIDS) and various viral hemorrhagic fevers have emerged worldwide, and diseases such as dengue fever and tuberculosis have re-emerged 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

Table 1 Main emerging & re-emerging viral diseases (past 30 years).

Disease	Region	Host
Rabies	Global	all mammalian
Yellow fever	South America, Africa	monkey-mosquitoes
Dengue F, Dengue HF	America, Asia, Africa	monkey-mosquitoes
Crimean-Congo HF	Africa, Asia, Eastern Europe	domestic animals, birds-mites
Japanese encephalitis	Japan, South East Asia	pigs-mosquitoes
Highly pathogenic avian influenza	America, Europe	birds
Hemorrhagic fever with renal syndrome	Asia,Europe	rodents
Hantaan virus pneumonic syndrome	America	rodents
Marburg disease	Europe, Africa	monkeys, bats?
Lassa fever	West Africa	rodents
B virus infection	USA	monkeys
Ebola hemorrhagic fever	Africa (Asia)	monkeys, bats?
Venezuelan HF	Venezuela	rodents
Argentine HF	Argentine	rodents
Bolivian HF	Bolivia	rodents
Rift valley fever	Africa	domestic animals-mosquitoes
Venezuelan encephalitis	Middle-South America	mosquitoes
AIDS (HIV type 1,2) Human Tcell lymphoma (HTLV1,2)	Africa (global)	monkeys?
Viral hepatitis (B, C, E) Human papilloma virus infection Idiopathic rash (HHV6, HHV7) Kabosi sarcoma (HHV8) Human parvo virus infection		Type E: pigs
BSE (vCJD): prions	UK	cattle
Nipah virus infection	Malaysia, Bang radish	fruit bats, pigs
Hendra virus infection	Australia	fruit bats, horses
West Nile fever	USA	Birds-mosquitoes
SARS	Asia, global	civets, Chinese raccoons?, bats
Highly pathogenic avian influenza(H5N1)	Asia, Europe, Africa	birds

* Black letters indicate zoonosis

such as methycillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), vancomycin-resistant *Staphylococcus aureus* (VRSA) and multi-drug-resistant *Mycobacterium tuberslosis*. 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. Since then, infectious disease control has become a hot topic at summit meetings.

3. Factors Behind the Occurrence and Spread of Zoonotic Diseases

The majority of zoonotic diseases can be traced to developing countries. The reasons for this include, first, increased contact with pathogens carried by wild animals in tropical rainforests and other natural habitats concomitant with a development of the rain forest resulting from human production activities (Ebola HF, Marburg disease, monkeypox); second, a disruption of ecosystems by rodents and other animals whose numbers have been elevated by increased human food productivity such as grain production (Bolivian HF, Lassa fever, Argentine HF, etc.); third, establishment of infectious diseases in cities of developing countries which is normally circulate 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 fourth, rapid spread of infections from developing to developed countries as a result of the rapid air transport of both people and animals during the diseases' latent period (Lassa fever, Marburg disease, SARS, etc.).

There are also factors contributing to zoonosis outbreaks in developed countries, such as keeping 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 efficiency in the form of intensive factory farming and rendering of animal parts not consumed by humans (animal offal) as sources of protein for feed (salmonella and campylobacter infections, BSE-vCJD, EHEC by 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 to humans through domestic animals such as horses and pigs, respectively.

The chances of coming into contact with infectious diseases 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 (Fig.1).

Even among wild animals, we may be facing new risks. For example, increasing environmental pollution may 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 morbilli virus, etc.), or environmental pollutants may elevate the frequency of virus mutations because the majority of

·Zoonosis from domestic animals, such as swine (Nipah virus), horses (Hendra virus), cattle (BSE) and chickens (HPAI) is more easily contagious to humans than that from wild animals and causes food-borne diseases.
·Once a pathogen invades a large factory farm of domestic animals, big outbreaks of epidemics occur easily.
·The mutation frequency is high when a virus spreads in a dense population of animals.

/Thus, the risk of zoonosis from domestic animals is increasing nowadays.

Fig. 1 Risks of domestic animals.
(a) Nipah virus (killing pigs in Malaysia) (by the CDC)
(b) HPAI (killing chickens in Japan)

persistent organic pollutants (POPs) may have a mutagenic potential. This kind of possibility suggests a need for changing paradigms and taking actions different from earlier measures for suppressing zoonoses and avoiding risks. Conservation medicine is a new approach to the control of zoonoses that incorporates the concept of environmental conservation in the consideration human and animal health. Thus, top down approaches from wildlife ecology and field science will become more important than bottom up research such as ordinal analysis of pathogens isolated from humans or domestic animals, which are the final hosts of the infections.

4. Zoonosis is a Warning to Humanity

The way in which zoonoses emerge and spread is changing in connection with the expansion of human production activities, the pursuit of economic efficiency, changing lifestyles, and so forth. In this respect, zoonoses have much in common with environmental pollutants such as PCBs, 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 ecosystems, 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 only itself are already proving to be bankrupt. What is needed is global cooperation among governments on countermeasures to zoonoses led by the WHO and Office International des Epizooties (OIE, now called "World Organization for Animal Health"). National governments should also be reminded that covering up or failing to report outbreaks, issuing "all clear" declarations prematurely, and 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 big outbreak (SARS in China, HPAIV in Southeast Asia, BSE-vCJD 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), which play 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 seven people, and 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 epidemic now involves Canada and Mexico. The USA is also finding it extremely difficult to suppress plague, which is endemic to arid Midwest regions (being transmitted between prairie dogs and fleas), rabies transmitted by bats and Hantavirus pneumonia syndrome (HPS) transmitted by wild rodents.

Meanwhile, the fact that SARS, which is thought to be of wild animal origin (now it is considered to be derived from a kind of bat corona virus), 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 has also spread from Asia to the Middle East, Europe, and Africa. The large number of countries involved, the big scale and persistence of infections in Southeast Asia, and the virulence that has enabled it to infect humans directly, have prompted the WHO to issue dire warnings about the dangers it poses. In addition, however, to conventional downstream, end-result-oriented infection countermeasures taken by the Ministry of Agriculture, Forestry and Fisheries (MAFF) and MHLW, targeting people and domesticated animals, in the 21st century, investigation is needed of zoonoses originating in wild animals 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 (Table 2).

Table 2 Strategy for zoonosis control.

Characteristics

/ The majority of recent world-threatening infectious diseases are zoonotic
/ Global outbreaks and re-emergence are occurring more easily
/ The cause of outbreaks is unclear and uncontrollable

Reason and countermeasures

/ The majority of serious zoonose were derived from wild animals
/ Thus, conservative bottom-up countermeasures targetting humans and liverstock are insufficient, but new top-down studies on the environment and ecology of wild animals and host-pathogen interactions are needed
/ Global measures for zoonosis control are necessary.

⬇

Strategy/tactics

/ Top-down (up-stream) studies on the ecology of sources of infection such as the environment, wildlife activities, host pathogen interactions, etc.
/ Establishment is needed of collaboration among field science, epidemics, ecology, contagious disease study and risk analysis
/ Establishment of a worldwide network on emerging and reemerging infectious diseases is necessary

5. 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 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 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 responsibility for controlling infectious diseases on an international level are the Geneva-based WHO for human infectious diseases, and the OIE, headquartered in France, for infectious diseases among animals and infectious diseases whose origins can be traced to foodstuffs. Since OIE decisions frequently directly affect the husbandry of domestic animals in all member countries (now 176 countries) and trade in foodstuffs of domestic animal origin, the OIE 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 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 – 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 get so-called informed consent), 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 such as the MHLW and MAFF. 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 infectious 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 (Fig.2).

6. Japan's New Zoonosis Countermeasures

After the postwar period of rapid economic growth in Japan (1950s), drastic changes in Japan's social system and values fueled trend towards nuclear families and a declining birthrate, and pets as companion animals came to serve as substitutes for people in the family. Then during the economic bubble of the 1980s, in place of the traditional species of pet animals, it became popular to import and keep so-called exotic animals. Japan's

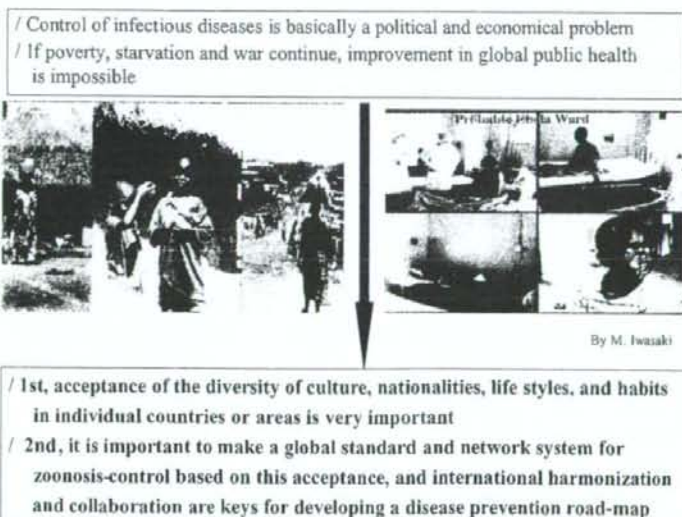

Fig. 2 The path to controlling zoonoses.

birthrate declined and the 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. Under the MHLW's emerging and re-emerging infectious disease research project, the status of animal importation was first investigated with the help of quarantine offices in 2000. Before that time, importation data on domestic animals and dogs was covered by the MAFF, and those on dogs, primates and honeybees were recorded by the Ministry of Finance for taxation. Data on other animals, however, were not covered by any of the ministries. The survey results indicated that about 4 million live animals were imported every year. They included about one million rodents, 2 million reptiles and 0.6 million birds and others (Table 3).

These changes in society and diversification in lifestyles prompted increasing concern over the possibility that novel zoonoses would emerge. Therefore, when the Infectious Diseases Control Law which was established about a hundred years ago was enacted (effective from 1999), in addition to diseases transmitted between people, zoonoses were also considered for the first time to be within the scope of the law and monkeys became subject to legal quarantine. Veterinarians were obligated to notify authorities when they diagnosed monkeys infected with Ebola HF or Marburg disease. Concomitant with an expanded application of the Rabies Prevention Law, cats, skunks, raccoons and foxes in addition to dogs also became subject to legal quarantine, as did monkeys. However, no other infectious zoonotic diseases and animal species were subject to regulation at that time, so when the Infectious Diseases Control Law came up for revision five years later, stronger measures were considered.

For the revision, data on outbreaks of zoonosis, statistics on imported animals and disease risk assessment were obtained and analyzed. An MHLW zoonotic disease study team carried out the first ever zoonosis risk analysis on imported animals in Japan. Briefly, for the prior five to ten years outbreaks of important zoonosis (about 50 diseases) worldwide were listed up (data obtained from the OIE, WHO and commercial data base of GIDEON) and each country's risk status was categorized from negligible to very high in regard to each zoonosis. Then, the number of imported animals from countries involved was classified from low (<100) to very high (>10,000). The first-step risk level was classified from negligible to very high by combining the risk status and number of imported animals in the country concerned as a lengthwise and crosswise matrix. Then quantitative risk points from zero to ten were calculated by combining the first-step risk level and severity of each zoonosis among human populations (Fig. 3). The total risk points of individual imported animal species were summed up for each zoonosis risk point from each country or area. If a certain animal species got high total risk points, importation of the animal was banned, and if the total points were relatively high, the animals were quarantined for one month or 21 days. For other animals, notification of animal importation became obligatory and certificate of purpose of breeding and pathogen-free certification by an authorized veterinarian were needed.

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 certification, and regulation according to risk level were

Table 3 Animals imported into Japan per year (2000).

Place	Number	Primates	Rodents	Other mammals	Birds	Bats	Reptiles	Amphibians	Others
Narita AP	2,338,504	4,200	1,021,800	20,840	271,847		1,019,817		
Kansai AP	1,082,972	401	27,233	7,541	12,817		971,437	59,707	3,836
Haneda AP	154,287				54,287				
Nagoya AP	154,249		5,293	509	146,635		1,812		
Fukuoka AP	87,020	5	36,030	702	10,090		24,956	15,219	
Kagoshima P	6,522		2,716	54	754		1,886	1,132	
Chitose AP	25						285		
Naha AP	20,805		13,970	279	3,912		2,644		
Sendai AP	20				20				
Yokohama P	17				17				
Osaka P	260			10			250		
Nagoya P	78			78					
Nagasaki P	10			10					250
Naha P	250								
Others						800			
Total	3,845,299	4,606	1,107,042	30,058	600,362	800	2,023,087	76,058	4,086

* AP means airport and P means port.

applied to all other animals apart from prairie dogs (for plague control) and civet cats (for SARS control) whose importation was already prohibited, and monkeys and carnivores already subject to legal quarantine. In other words, unlike previous revisions which tended to simply increase legal animal quarantines, the new revision applied import bans to certain species, and according to the risk level, introduced stronger measures against imported animals and indigenous wildlife (migratory birds, crows, etc.) including surveillance systems, investigation of animals in the event of outbreaks 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 importation 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 (Fig 4).

With respect to wild and domestic animals within Japan, everyday surveillance is important, 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 or zones, 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 governments, between the MAFF and MHLW, and between doctors and veterinarians.

Figure 4 bottom is posters for zoonosis control by the new regulation of animal importation.

Left: Importation ban of mastomis and chiropters.

Middle: Flow chart of the newly established animal importation procedure.

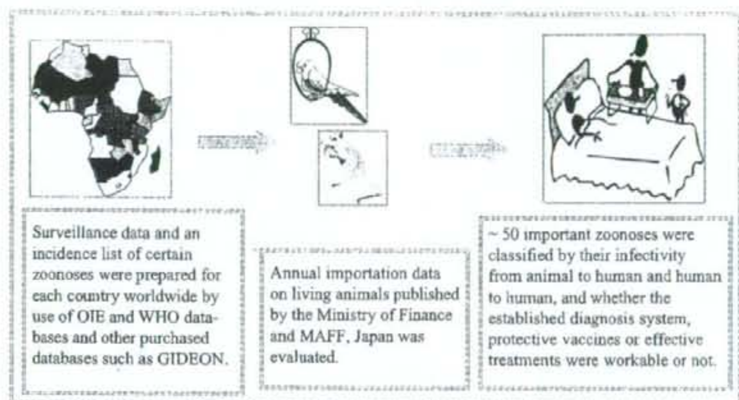

Fig. 3 Risk assessment of zoonoses in Japan.
1st : Risk status of the zoonosis concerned in each region or country
2nd : Annual importation data on living animals
3rd : Assessment of each zoonosis for its severity to humans

/ Importation ban of high risk animals: chiropters, mastomis (Nov. 2003)
prairie dogs, civets have already been banned for plague and SARS control, respectively.
/ Legal quarantine of monkeys, dogs, cats, raccoons, foxes and skunks
/ Shigella dysentery, TB in monkeys designated notifiable diseases for veterinarians (VMD)
/ Notification required of dog echinococcosis, West Nile virus infection in birds by VMD
/ Other imported animals required to get permits from the MHLW quarantine office
(2005: health certificates, breeding records for rodents, birds, etc.)
/ Active surveillance, enforcement of laws on discarding zoonotic contaminants
/ H5N1 HPAI declared a designated disease (2006)
/ Almost all zoonoses in humans declared notifiable diseases by medical doctors (MD)

Fig. 4 Risk management for zoonosis control in Japan.

Table 4 Effects of new zoonosis control on risk avoidance.

Number of imported animals in 2000 (investigated by the MHLW research project)

Total	Primates	Rodents	Carnivores	Birds	Chiropters	Reptiles	Amphibians	Others
3,845,299	4,606	1,107,042	30,058	600,362	800	2,023,087	76,058	4,086

Number of imported animals after new regulations implemented
(2005 Sep. ~ 2006 Aug., 2006 Sep. ~ 2007 Aug.) IANOS data (imported animal notification operation system)

	2005~2006	(per 2000)	2006~2007	(per 2000)
Mammalian	511,348	(44.7%)	432,819	(37.9%)
Avian	112,986	(18.8%)	100,961	(16.8%)

Origin of imported animals after new regulation

	Mammalian		Avian	
	2005~2006	2006~2007	2005~2006	2006~2007
Wild	4	1	3,230	8,127
Bred	511,29 (99.9%)	43,288 (100%)	109,693 (97.1%)	92,830 (91.9%)
Unknown	53	30	63	4

* unknown refers to pet animals (disposed), and the wild animals were for zoos (bears, flamingos, raptors)

Right: New compulsory notification for importation of pet animals such as birds and rodents.

7. Validation of the Effect of New Zoonosis Control on Risk Avoidance

Since the imported animal notification system was a precautionary measure under risk assessment, verification of the system was conducted two years after the introduction of the new zoonosis control system, i.e., for notification of imported animals. From September 2005 (the starting point of the new measures) to August 2006 and from September 2006 to August 2007, the number of annual live animal imports was calculated and compared with that of 2000.

It is clear that the number of imported animals drastically decreased, both mammalian and avian. Moreover, the origin of animals shifted from wild caught to purposefully bred (Table 4). This was reflected in changes in the order of exporting countries, i.e., before 2005 many animal species, including rodents, came from the Middle East, Near East Asia and African countries, but after 2005 the exporting countries shifted to Far East Asia, European countries and the USA (data not shown). It appears that this risk assessment and precaution measures worked effectively to avoid the risk of zoonosis outbreaks.

8. Prospects

Recently the main cause of human infectious diseases and public health threats has been considered "emerging and re-emerging diseases." These infectious diseases are mainly derived from pathogens from animals (zoonosis) and their products (food-borne infections). The zoonoses worldwide were reviewed by their origin, environment and relationship to human activities in both developing and developed countries. Emerging zoonosis is a kind of warning with regard to human activities and we need to create ubiquitous scientific zoonosis control methods acceptable to almost all countries of the world. The WHO and OIE are working as internationally responsible organs for disease control in humans and domestic animals, respectively. I have explained herein a path to global zoonosis control and our recent experiences in risk assessment and measures for zoonosis control in Japan. The prospect' for zoonosis control are based on the Precautionary Principle and crisis management which should be implemented by cooperation between central and local governments, between the MAFF and MHLW, and between medical doctors and veterinarians, as well as with collaboration between countries and international organs.

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Ligation-mediated amplification for effective rapid determination of viral RNA sequences (RDV)

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Abstract

Background: Emerging infectious diseases pose a significant risk to public health. Methods for rapid detection of pathogens are needed to effectively treat these diseases. Recently, we developed new methods for the rapid determination of viral RNA sequences, RDV ver1.0 and ver2.0. We demonstrated that these methods were able to simultaneously detect cDNA fragments of many different viruses without using sequence specific primers. However, some species of viruses, including the Yokose virus (YOKV), a flavivirus, could not be detected using the conventional procedures.

Objective: The RDV method was further modified to reduce the candidate PCR primer sets.

Study design: Primer sets were reduced to 256 sets in the improved RDV ver3.0, and theoretically, all viral cDNA fragments ligated by two kinds of adaptors after digestion by two restriction enzymes could be amplified in the PCR step for direct sequencing.

Results: We succeeded in obtaining 118 YOKV cDNA fragments of the 141 sequence fragments. The cDNA fragments covered diverse range of viral genome.

Conclusion: We were able to reduce the combinations of PCR primer sets used in the RDV method. This RDV method ver3.0 has a potential to detect viral cDNA fragments of both known and unknown RNA viruses rapidly and conveniently.

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Keywords: RDV; Rapid determination; Direct sequence; Yokose virus; Virus discovery

1. Introduction

Recently, we developed a method for sequence-independent detection of RNA viruses, the "rapid determination of RNA virus (RDV) method" (Mizutani et al., 2007). In the RDV method, viral genome sequences are obtained without sub-cloning into plasmid vectors. Nucleic acid sequences of severe acute respiratory syndrome coronavirus, murine hepatitis virus, West Nile virus, Japanese encephalitis virus, and Dengue virus were successfully detected in culture supernatants from virus-infected cells by RDV ver1.0, which is our original method for detecting RNA viral genomes (Mizutani et al., 2007; Kihara et al., 2007). To increase sensitivity over RDV ver1.0, the sequence-independent amplification step was improved. By using the improved method, RDV ver2.0, avian paramyxovirus was detected in the allantoic fluid of embryonated chicken eggs (Sakai et al., 2007). A new adenovirus was successfully detected in the culture supernatant of primary kidney cells originating from a fruit bat using this RDV method (RDV-D) (Maeda et al., 2008). RDV methods have the potential to become standard methods for the

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detection of both known as well as newly emerging unknown viruses in humans and animals.

For direct sequencing after amplification of cDNAs in RDV ver1.0 and ver2.0, we use specially designed primer sets in which 6 nucleotides consisting of the CC (HaeIII-digested sequence) and four variable nucleotides are added to the 3′ end of the adapter sequence. Therefore, very large numbers of combinations of primer sets ($4^4 \times 4^4 = 65{,}536$ sets) are theoretically necessary to detect all the viral genomes in a sequence-independent manner (Mizutani et al., 2007). To avoid this level of complexity, we conventionally used the primer sets fixed the 5′ end of two nucleotides in the four variable nucleotide region to AG for the forward primer or GG for the reverse primer. However, we found that some viral species are difficult to detect using the limited combinations of primer sets. For example, the Yokose virus (YOKV) was isolated from a bat in Japan in 1971, and it was found to be an Entebbe bat virus, genus *Flavivirus*, in the family *Flaviviridae*. Although the full genome sequence has been published recently (AB114858), little is yet known about the characteristics of the virus (Tajima et al., 2005). We attempted to detect the YOKV sequence in the culture supernatant from virus-infected Vero cells using the procedure of RDV ver1.0 and ver2.0, but this failed. Therefore, in this study, we further improved the RDV method to detect more virus species, in particular by modifying the adaptor ligation step, to reduce the candidates of PCR primer sets for direct sequencing. This RDV ver3.0 was able to detect over 100 cDNA fragments of YOKV.

2. Methods

2.1. Cells and virus

Vero cells were maintained in Dulbecco's modified Eagle's medium with 5% fetal calf serum, penicillin, and streptomycin. The Oita-36 strain of YOKV was kindly provided by Dr. Tomohiko Takasaki (National Institute of Infectious Diseases of Japan). The virus was propagated in Vero cells. At 2 days post-infection, the infectious fluid was harvested. Cellular debris was removed by low-speed centrifugation ($2000 \times g$, 15 min, 4 °C) and the resulting supernatant was collected.

2.2. Design and scheme of RDV ver3.0

The RDV ver3.0 method includes the four procedures described below (Fig. 1).

2.2.1. RNA extraction

Viral RNA was extracted from the infectious supernatant containing $10^{5.5}$ $TCID_{50}$ of viruses by using a Total RNA isolation mini kit (Agilent Technology, USA) in accordance with the manufacture's instruction.

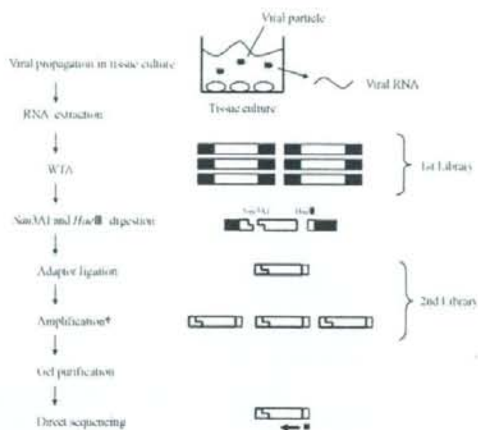

Fig. 1. Overall scheme for RDV ver3.0. WTA, whole transcriptome amplification; (†) with specially designed primer sets as shown in Fig. 2B.

2.2.2. Construction of first cDNA library

A whole transcriptome amplification system (WTA; Sigma–Aldrich, Saint Louis, MO, USA) was used to amplify viral double-stranded cDNA in accordance with the manufacture's instruction. PCR was performed as described in previous paper (Mizutani et al., 2007).

2.2.3. Second cDNA library

After the first cDNA library purification using the MonoFas DNA isolation system (GL Science, Japan), DNA was digested with 40 U of HaeIII (Takara Bio Inc., Japan) and Sau3AI (Takara Bio Inc.) at 37 °C for 30 min, and then the digested DNA was again purified using MonoFas. For construction of the second cDNA library, 2.5 μl of DNA solution, 2.5 μl of distilled water, 2.5 μl of sticky-ended adaptor, Adaptor-Sse83871 (10 μM) and blunt-ended adaptor, Adaptor-NotI (10 μM) were mixed (Fig. 2A). Ligation-convenience kit (Nippon Gene, Japan) was used for adaptor ligation. The DNA solution and 10 μl of ligation mix were reacted at 16 °C for 30 min, and the DNA was isolated using MonoFas. The second cDNA library was amplified by PCR using specially designed primer sets, and the forward primers in which six nucleotides included GATC (Sau3AI-digested sequence) and two variable nucleotides were added to the 3′ end of the Adaptor-Sse83871 sequence, and the reverse primers in which four nucleotides included CC (HaeIII-digested sequence) and two variable nucleotides were added to the 3′ end of the Adaptor-NotI sequence (Fig. 2B). PCR was performed as described in Sakai et al. (2007).

2.2.4. Direct sequencing

After electrophoresis of PCR products on agarose gels, the bands over 150 bp in length were excised, and DNA was extracted from the gel using the MonoFas. Direct sequencing was performed using the forward or reverse primer.

(A)

Adaptor-Sse83871

5'-ATCAGCGGGTTTAAACGGGCCCTCTAGACGATCAGGATCCTGCAGG-3'
3'-TAGTCGCCCAAATTTGCCCGGGAGATCTGCTAGTCCTAGGACGTCCCTAG-5'

Adaptor-NotI

5'-CACTATAGGGAGACCCAAGCTGGCTAGCAATTCGGCGGCCGCGGATCC-3'
3'-GAGTGATATCCCTCTGGGTTCGACCGATCGTTAAGCCGCCGGCGCCTAGG-3'

(B)

S1	5'- gacgatcaggatcctgcagggatc **aa**-3'	N1	5'- aattcggcggccgcggatcccc **aa**-3'
S2	gacgatcaggatcctgcagggatc **at**	N2	aattcggcggccgcggatcccc **at**
S3	gacgatcaggatcctgcagggatc **ag**	N3	aattcggcggccgcggatcccc **ag**
S4	gacgatcaggatcctgcagggatc **ac**	N4	aattcggcggccgcggatcccc **ac**
S5	gacgatcaggatcctgcagggatc **ta**	N5	aattcggcggccgcggatcccc **ta**
S6	gacgatcaggatcctgcagggatc **tt**	N6	aattcggcggccgcggatcccc **tt**
S7	gacgatcaggatcctgcagggatc **tg**	N7	aattcggcggccgcggatcccc **tg**
S8	gacgatcaggatcctgcagggatc **tc**	N8	aattcggcggccgcggatcccc **tc**
S9	gacgatcaggatcctgcagggatc **ga**	N9	aattcggcggccgcggatcccc **ga**
S10	gacgatcaggatcctgcagggatc **gt**	N10	aattcggcggccgcggatcccc **gt**
S11	gacgatcaggatcctgcagggatc **gg**	N11	aattcggcggccgcggatcccc **gg**
S12	gacgatcaggatcctgcagggatc **gc**	N12	aattcggcggccgcggatcccc **gc**
S13	gacgatcaggatcctgcagggatc **ca**	N13	aattcggcggccgcggatcccc **ca**
S14	gacgatcaggatcctgcagggatc **ct**	N14	aattcggcggccgcggatcccc **ct**
S15	gacgatcaggatcctgcagggatc **cg**	N15	aattcggcggccgcggatcccc **cg**
S16	gacgatcaggatcctgcagggatc **cc**	N16	aattcggcggccgcggatcccc **cc**

Adaptor sequence Sau3AI- digested sequence Adaptor sequence HaeIII - digested sequence

Fig. 2. The design of adaptors and primer sets used in RDV ver3.0. (A) Adaptor-Sse83871 contains sticky-end structures digested with Sau3AI, whereas Adaptor-NotI contains blunt-end structures digested with HaeIII. (B) All primers used in RDV ver3.0. Bold letters indicate 2-variable nucleotides.

3. Results

In RDV method ver3.0, different adaptors were ligated at the 5′ and 3′ end of viral dsDNA. The ligation enzyme was changed to the ligation mix in the Ligation-convenience kit (Nippon gene) because this increased the efficacy of ligation (data not shown). The S1–S16 forward primers and N1–N16 reverse primers were used in the second cDNA amplification step for direct sequencing (Fig. 2B). Using the RDV ver3.0, we successfully identified West Nile virus and Dengue virus type 2, which were used in previous our study (Mizutani et al., 2007) (data not shown). The RDV ver3.0 method was performed using extracted RNA from the culture supernatant obtained from YOKV-infected Vero cells. The 256 PCR

Fig. 3. Location of viral cDNA fragments on YOKV genome amplified using the RDV ver3.0. Column A shows 10 cDNA fragments expected to be detected in RDV ver3.0 (over 150 bp cDNA fegments digested by Sau3AI and HaeIII). Columns B–E show YOKV cDNA fragments detected in RDV ver3.0. Column B shows 8 of 10 cDNA fragments as shown in A. Column C shows cDNA fragments partially digested with HaeIII or Sau3AI. These cDNA fragments contained undigested sequences by the restriction enzymes. Column D shows amplicons detected as 2 or 3 ligated cDNA fragments. Column E shows cDNA fragments not containing recognition sequences of restriction enzymes at the ends.

Table 1
Number of sequence DNA fragments detected in RDV ver3.0

Forward primer	Number of sequence DNA fragment	Number of expected DNA fragment
S1	11/11 (100) [a]	2 (2) [b]
S2	21/21 (100)	2 (2)
S3	3/3 (100)	0 (0)
S4	5/8 (62.5)	0 (0)
S5	11/11 (100)	2 (2)
S6	18/18 (100)	1 (1)
S7	1/3 (33.3)	0 (1)
S8	8/11 (72.7)	0 (1)
S9	11/11 (100)	0 (0)
S10	11/16 (68.7)	0 (0)
S11	5/6 (83.3)	0 (0)
S12	3/6 (84.17)	0 (0)
S13	4/6 (66.6)	1 (1)
S14	0/0	0 (0)
S15	0/1 (0)	0 (0)
S16	5/12 (41.6)	0 (0)
Total	118/141 (83.6)	8 (10)

[a] Number of YOKV cDNA fragments/number of sequence DNA fragments (percent).

[b] Number of detected YOKV cDNA fragments of (number of YOKV cDNA fragments expected to be detected from complete nucleotide sequence).

products were processed by agarose gel electrophoresis. A total of 141 fragments were sequenced by the direct sequencing method, but sequences of 10 fragments were not obtained. One hundred eighteen exhibited high degrees of homology with the nucleic acid sequence of YOKV. Twenty-two DNA fragments were identified as mammal ribosomal RNAs (data not shown). The locations of the PCR fragments found in the YOKV genome are shown in Fig. 3. From the complete nucleotide sequences of YOKV, there are 10 cDNA fragments over 150 bp with HaeIII- and Sau3AI-digested ends (Fig. 3 column A), which are expected to be detected using RDV ver3.0, and eight fragments were detected (Table 1 and Fig. 3 column B). Nine were obtained as partial digested viral cDNA fragments by HaeIII or Sau3AI (Fig. 3 column C). In addition, we found two or three ligated viral cDNA fragments (Fig. 3 column D) and cDNA fragments not containing recognition sequences of restriction enzymes at the ends (Fig. 3 column E). The limit of detection of RDV ver3.0 was approximate 10^4 copies of viral genomic RNA as a starting material when using YOKV (data not shown).

4. Discussion

In this study, we succeeded in reducing the candidates of PCR primer sets for direct sequencing by improving the adaptor ligation step in RDV method. Theoretically, by using all combinations of the S1–S16 forward and N1–N16 reverse primers, all viral cDNA fragments ligated by two kinds of adaptors could be amplified in the direct sequencing step. Therefore, our newly developed RDV ver3.0 has been demonstrated to be superior in detecting unidentified viruses within at most 256 PCR reactions.

After direct sequencing, we found that viral DNA fragments could be amplified by PCR with a primer set in which one of two variable nucleotides in forward or reverse primers are matched to the target fragment. We obtained amplicons containing two or three ligated DNA fragments and also obtained viral DNA fragments partially digested with HaeIII or Sau3AI (Fig. 3). As shown in Fig. 3, many DNA fragments, covering a diverse range of viral nucleotide sequences, were detected. This suggests that the sequenced fragments detected by RDV ver3.0 can be used for the design of primers to determine complete viral nucleotide sequences for long PCR when unknown viruses are detected.

In this study, we were able to reduce the combinations of PCR primer sets used in the RDV method to a very large degree. This RDV method ver3.0 promises to greatly improve sequence-independent detection of RNA viruses especially when emerging virus disease occurs.

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Prevalence of herpes B virus genome in the trigeminal ganglia of seropositive cynomolgus macaques

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Summary

Herpes B virus infection is almost asymptomatic in macaques (*Macaca* spp.), which are the natural hosts of this pathogen, but is the cause of high mortality in humans. Reactivation of the latent virus in the trigeminal ganglia (TG) results in the shedding of infectious particles into the oral mucosal membrane. Saliva contaminated with the reactivated virus from the ganglia of the natural host is considered to be important for viral transmission to humans and other monkeys. In the present study, we investigated the prevalence of the herpes B virus genome in the left and right TG of seropositive asymptomatic cynomolgus macaques. The latent virus genome was detected using a polymerase chain reaction and microplate hybridization assay. We found that the virus DNA was present in one or both TG of 12 of the 30 macaques (40%) tested, with the virus being detected from both TG in five of the 12 macaques and from a single TG in the remaining seven.

Keywords Herpes B virus; trigeminal ganglia; seropositive; cynomolgus macaque; latent infection

Herpes B virus disease in humans is caused by infection with the herpes B virus (*Cercopithecine herpesvirus 1*), which is harboured in the natural hosts, Asian macaques including rhesus (*Macaca mulatta*) and cynomolgus (*Macaca fascicularis*) macaques (Keeble *et al.* 1958, Davenport *et al.* 1994, CDC 1998). The infection results in severe encephalomyelitis in humans with a mortality of over 50%, whereas almost no symptoms are seen in the natural hosts (Weigler 1992). Herpes B virus is transmitted mainly by contact with contaminated excreta from infected macaques (Cohen *et al.* 2002). Asian macaques are important laboratory animals because of their physiological and morphological similarity to humans with inevitable frequent contact between animal care workers and macaques. Therefore investigation of the herpes B virus prevalence in Asian macaques is important for effective health and safety controls.

The herpes B virus, like other alphaherpesviruses, such as herpes simplex virus type 1 (HSV-1), HSV-2 and varicella-zoster virus (VZV), has a high neurotropism (Whitley & Hilliard 2001). The virus invading the nerve terminals is transported by a retrograde flow to the sensory ganglion cell bodies and then establishes a latent status in the cells (Espana

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1973, Boulter & Grant 1977, Lees 1991, Weigler 1992). As reported in human herpesviruses, reactivation of the herpes B virus leads to the shedding of infectious particles from mucosal membranes following anterograde transport in the sensory neurons (Zwartouw & Boulter 1984, Weigler 1992, Weigler *et al.* 1993, 1995, Huff *et al.* 2003). The virus particle is excreted at the peripheral sites controlled by the latently virus-infected ganglion. The reactivated herpes B virus from the trigeminal ganglia (TG) and lumbosacral ganglia is suggested to be propagated and excreted from the oral or ocular, and the genital mucosal membrane, respectively (Boulter 1975, Weigler *et al.* 1995).

More than 40 cases of herpes B virus infection in humans have been reported since the first report by Sabin and Wight (Sabin & Wright 1934, Engel *et al.* 2002). It has been suggested that the virus is transmitted mainly by bites and/or by contact with contaminated saliva from virus-infected macaques (Cohen *et al.* 2002). We investigated the prevalence of the herpes B virus in the TG of the cynomolgus macaque, which is one of the most frequently used Asian macaques. We examined seropositive macaques, which were suggested to be latently infected with the virus in some ganglia. We postulated that the virus was present in one or both TG and was dependent on the site of original virus infection. The left and right TG were removed separately from macaques and DNA was extracted. The genome of the herpes B virus in the TG was detected and identified by a polymerase chain reaction (PCR) and microplate hybridization assay, as reported previously (Oya *et al.* 2004).

Materials and methods

Animals

A total of 30 cynomolgus macaques were investigated for the presence of viral DNA. Ten (group A) and 20 (group B) macaques bred in China and imported in 1999 and 2000, respectively were sourced from contract research laboratories in Japan. All macaques were between two and six years old (the majority of macaques were 2–4 years old), and showed no clinical symptoms. Antibody to herpes B virus was detected from all macaque sera by enzyme-linked immunosorbent assay. Table 1 shows some characteristics of the macaques.

Sample preparation

The left and right TG were removed separately at necropsy from the macaques following euthanasia using an overdose of intravenous injection of pentobarbital and simultaneously 5 mL of whole blood was collected. The peripheral blood mononuclear cells (PBMC) were collected from the buffy coat after centrifuging at 1850 **g** for 5 min. Each TG and PBMC was immersed in 400 μL of Tris-EDTA-SDS (TES) buffer (10 mmol/L Tris–HCl [pH 7.8], 10 mmol/L EDTA, 0.6% [w/v] sodium dodecyl sulphate [SDS]). The tissue samples were stored at 4°C until DNA extraction. All experimental procedures were approved by the Institutional Animal Care

Table 1 Characteristics of cynomolgus macaques used in the present study

Group	Animal no.	Sex	BW (kg)
A	A1	M*	4.67
	A2	M	4.99
	A3	M	4.01
	A4	M	3.85
	A5	M	4.22
	A6	F†	3.59
	A7	F	3.24
	A8	F	2.96
	A9	F	3.53
	A10	F	3.25
B	B1	M	3.65
	B2	M	2.90
	B3	M	3.05
	B4	M	2.45
	B5	M	2.50
	B6	M	3.30
	B7	M	3.20
	B8	M	2.85
	B9	M	3.10
	B10	F	3.00
	B11	F	3.15
	B12	F	2.90
	B13	F	2.60
	B14	F	2.60
	B15	F	2.35
	B16	F	2.65
	B17	F	3.00
	B18	F	2.55
	B19	M	3.05
	B20	M	3.25

*M, male
†F, female

and Use Committee of the Graduate School of Agricultural and Life Sciences, the University of Tokyo.

DNA extraction from TG and PBMC

The TG samples and PBMC samples were digested with 570 μg/mL of proteinase K (Promega, Tokyo, Japan) at 55°C for 5 h in TES buffer. The DNA was extracted and purified with phenol and chloroform followed by precipitation in ethanol. The DNA was dissolved in 200 μL of TE buffer (TES buffer without SDS) and stored at 4°C.

Plasmid as a positive control and a probe

A cloned 2.6 kb SalI-EcoRI fragment containing the US5, US6 and a part of US7 derived from the herpes B virus SMHV strain (SMHV/pBV-DNA) was kindly provided by Dr Akio Yamada of the National Institute of Infectious Diseases of Japan (Bennet *et al.* 1992).

Polymerase chain reaction

We amplified the C region or E region, which consisted of most of US5 and the sequence between US5 and US6, and the 3′-end of US5 and the sequence between US5 and US6, respectively, as described previously (Oya *et al.* 2004). Amplification of the C region and E region was carried out with an HB2A and HB2B primer set, and HB3A and HB2B primer set, respectively (Table 2). Polymerase chain reaction was performed essentially as described previously but with some modifications (Oya *et al.* 2004). We prepared a 100 μL PCR mixture containing each primer at 0.5 μmol/L, reaction buffer, a mixture of dNTPs (0.2 mmol/L each), 2.5 units of *Ex Taq* polymerase (Takara, Shiga, Japan), 2.5% (v/v) DMSO (Merck, Tokyo, Japan) and 10 μL of template. Non-diluted or 1:10 diluted DNA solution was used as the template. Polymerase chain reaction was carried out as described below; initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 2 min, annealing at 65°C for 3 min and extension at 72°C for 4 min and final extension at 72°C for 10 min.

Microplate hybridization

The PCR products were processed by extraction with phenol and chloroform, precipitation in ethanol, and dissolved in 100 μL of TE buffer. The DNA solution was further purified using a spin column (SUPREC™-02; Takara, Shiga, Japan) according to the manufacturer's instructions. Microplate hybridization was carried out as described previously (Oya *et al.* 2004). The C region amplicon derived from the SMHV strain was used as the probe.

Statistical procedures

Fisher's exact test and the χ^2-test with Yates' correction ($P < 0.05$) were used, where appropriate, for comparison of the distribution of virus-positive macaques.

Results

Detection and identification of herpes B virus DNA in TG

Polymerase chain reaction and microplate hybridization assay were performed to investigate the prevalence of the latent infection of the herpes B virus in the left and right TG of 30 seropositive cynomolgus macaques. Herpes B virus DNA was identified in the TG of 12 of the 30 macaques, five in group A and seven in group B (Table 3). In terms of gender, of the 16 male and 14 female macaques, the virus was detected from the TG of five male and seven female macaques. There was no statistically

Table 2 Herpes B virus primers used in the present study

Primer	Primer sequence	Positions*
HB2A sense	5′-CCGCGCTCGCCACGGACACCA-3′	411–431
HB3A sense	5′-CCTGCACCGGTGCTGTAGACG-3′	643–663
HB2B antisense	5′-ATCGCGCGCCGGACCGATCGT-3′	1072–1052

*Nucleotide positions referred to the E2490 sequence number described by Smith *et al.* (1998) (GenBank accession no. AF083210)

Table 3 Viral DNA distribution in the trigeminal ganglia (TG) of cynomolgus macaques

Group	Sex	No. of macaques in which virus DNA was detected			
		Left TG	Right TG	Both TG	Total
A	M*	0	0	1	1
	F†	1	3	0	4
B	M	0	2	2	4
	F	0	1	2	3
Total		1	6	5	12

*M, male
†F, female

significant gender difference in the number of virus-positive macaques. It was confirmed that the virus was retained in a latent state in 30 seropositive cynomolgus macaques by detection of no herpes B virus genome in the PBMC of seropositive cynomolgus macaques using PCR (data not shown). The distribution of virus between the left and right TG is also shown in Table 3. There was no statistically significant difference between the number of macaques in which the virus DNA was detected in either one or both TG.

Hybridization of detected DNA with the SMHV strain probe at 56°C and 42°C

The amplicons from two macaques of group A (A6 and A7) were hybridized with the SMHV probe at both 56°C and 42°C, whereas those from the other three (A2, A9 and A10) were hybridized at only 42°C. However, all PCR products from the TG of the group B macaques (B2, B3, B4, B9, B12, B14 and B16) were hybridized with the probe at both temperatures.

Discussion

We investigated the prevalence of the herpes B virus in the left and right TG of seropositive cynomolgus macaques by a PCR and microplate hybridization assay. In the present study, a total of 30 macaques imported in two batches from China were investigated. The herpes B virus DNA was detected and identified in the TG from 50% and 35% of the group A and group B macaques, respectively, indicating that 40% of the seropositive macaques had virus DNA detectable in their TG. Other reports have shown that the viral DNA was detected by PCR from 27.8% and 10% of seropositive rhesus and Japanese macaque TG, respectively (Weigler *et al.* 1993, Ohsawa *et al.* 2002). It is suggested that the macaques with no detectable viral DNA in their TG may have the virus in other ganglia and/or that the quantity of viral DNA used in each PCR would be smaller than the detectable limits of each PCR assay.

Our results suggest that almost 60% of the herpes B virus genome-positive macaques examined in the present study were infected with the herpes B virus in a single TG. In contrast, studies of HSV-1 and VZV have shown that the virus DNA was detected from both TG in most of the patients (Mahalingam *et al.* 1990, Furuta *et al.* 1992, Cohrs *et al.* 2000). Cohrs *et al.* reported that HSV-1 DNA was detected in the single TG of one (8.3%) out of 12 human subjects in which the virus DNA was detected in TG. It has been reported that VZV DNA was detected in both TG of all (100%) of 17 (Cohrs *et al.* 2000) and all of seven (Mahalingam *et al.* 1990) human subjects in which virus DNA was detected. Furuta *et al.* reported that VZV DNA was detected in a single TG of one (16.7%) of six human subjects in which virus DNA was detected in TG (Furuta *et al.* 1992). These contrasting results may reflect differences in the region of the body where the hosts were exposed to the virus, and/or the ability of the virus to replicate and spread in the host from the site of primary infection. However, we cannot exclude the possibility that the latent virus in either TG is reactivated, and that the resulting infectious particles then spread to the other TG. Mainly elderly persons with a serious clinical history, which might be induced by immunosuppression, have been investigated in human studies, whereas apparently healthy young macaques (the majority of macaques were 2–4 years old) were used for our examination. In elderly subjects with a compromised immune system, the human herpesviruses may recrudesce more frequently than the herpes B virus in the young animals examined in the present study.

Further investigation of the prevalence of the herpes B virus in various Asian macaque species would lead to a better understanding of virus transmission between host species and conclusions would be facilitated by the determination of viral nucleotide sequences.

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第2章

食の安全・安心における獣医師の役割

05

日本における牛海綿状脳症(BSE)のリスク評価

わが国では，食品の安全性を行政が保証し，国民は批判せずにそれを受け入れる方式で安全神話を作り上げてきた。また出来上がった安全神話に立脚しシステムの検証を行うこともしない。したがって一度安全神話が崩れるとパニックが起こり，改めてシステムを再検討することになる。BSEパニック後に導入されたシステムがリスク分析であり、リスク評価とリスク管理を分離するため内閣府に食品安全委員会が設置された。プリオン専門調査委員会は医師と獣医師等12名からなり，プリオンに関するリスク評価を行う責務を負った。専門委員会が行うリスク評価に関する審議はすべて公開で，審議経過と結果はホームページに公開される。

ここではこれまで委員会で行われたBSEのリスク評価を中心に，この新しいシステムの有効性と問題点について論じる。

1．リスク分析とリスク評価

食品の安全性は科学的にどのように保証されるのであろうか？　安全性と正反対の言葉に「リスク」という概念がある。リスクの減少は安全性の増加に比例する。現在では食品の科学的安全性はリスク評価というステップを経て確保される。リスク評価はリスク分析という自然科学と社会科学が一体となった新しい学問分野に含まれる。リスク分析はリスク評価，リスク管理，リスクコミュニケーションという三要素からなっている。科学者による科学的・中立的なリスク評価をもとに，行政(リスク管理機関)が費用対効果やリスクのトレードオフ等を考慮して管理措置や基準などの施策を決定し，消費者やステークホルダー(すべての利害関係者)に対して説明と同意という，リスクコミュニケーションを介して施策の実現を図ろうという考え方である。

科学的なリスク評価は具体的にどのように進めるのであろうか？　リスク評価はヒトに危害を与える原因となる因子を同定することから始まる。畜産物を介してヒトに危害を与える要因には，病原微生物(プリオンを含む)，飼料添加物，食品加工時の添加物や生物製剤など様々なものがある。こうした危害物質(ハザード)の1つ1つについて，リスクのシナリオを作成する(リスクプロファイリング)。すなわち，最終的な畜産物由来食品に危害因子がどのレベルで残留するか，それを食べた場合にどの程度の有害作用を示すか？　そのようなケースがどのくらいの頻度で起こる可能性があるのか？　といったことを分析する。有害作用はヒトでの疫学調査や動物実験のデータ等を基に評価する。農場から食卓までの安全性の一括管理や，食品加工時の検査等のリスク低減措置なども考慮し，できるだけ総合的，定量的に評価する。こうしたリスク分析手法は近年，先進国で整備されたシステムである。しかし，わが国は経験が浅く，

1）リスク評価機関による説得性のある客観的な科学的評価の実施と検証

2）リスク管理機関による透明性のある施策決定と説明責任の遂行
3）自己責任を伴う商品の選択とリスク評価・管理措置に対する意見表明

など，リスク評価，管理，消費のどの分野も，まだ発展途上である。

2．日本のBSE対策と疫学

（1）日本のBSE対策，vCJD対策[1)]

BSEの主な原因となった肉骨粉に関しては1996年4月に牛への使用禁止通知が出され，2001年10月には牛の飼料への肉骨粉の使用禁止，特定危険部位(SRM)の焼却，牛由来肉骨粉の焼却が法制化された。牛用飼料に関しては交差汚染防止のため製造工程の完全分離(2004年末)，輸入飼料規制強化(2005年)を行い，輸入飼料の末端までの届出・記録保管を義務付けた。

牛からヒトへの感染防止のため食肉や内臓などの牛由来加工品に関してはと畜牛全頭検査，陽性牛焼却処分(2001年)，SRMの除去と焼却(全頭，2001年)，また脊柱をSRMに含めた(2004年)。と畜場におけるBSE検査は2005年から21カ月以上にすることとしたが，経過措置として3年間全頭検査を続けることになった。牛由来成分を含む医薬品に関してはイギリス牛由来医薬品輸入・製造禁止(1996年)，高リスク国牛由来材料の使用禁止(2000年)，すべての牛のカテゴリーⅠ，Ⅱ組織(高いリスクを持つ可能性のある組織)の使用禁止(2001年)，低リスク国牛由来Ⅲ，Ⅳ組織(リスクの無視できる組織)由来材料のみ使用可(2001年)と変更された。

ヒトからヒトへの感染防止は基本的にリスクのあるヒトからの輸血と臓器移植を禁じている。1980〜96年の間，イギリスに6カ月以上滞在した者の献血禁止(1999年)，1980年以降イギリス，フランス，ドイツ，スイス，アイルランド，ポルトガル，スペインに6カ月以上滞在した者は献血，臓器提供禁止(2001年)，滞在国を全欧州BSE陽性国に拡大(2003年)，わが国で一例の変異型クロイツフェルト・ヤコブ病(vCJD)患者が見つかり，イギリスに24日間滞在したことが明らかになったため，1996年までにイギリスに1日以上滞在した者は献血・臓器提供禁止となった(2005年)。

このほか，2001年10月からサーベイランスとして24カ月齢以上の異常牛の検査，2002年4月に個体識別のための耳標取り付け，2003年12月から生産段階のトレーサビリティ，2004年12月から流通過程のトレーサビリティ制度を法制化した。2003年4月から死亡牛検査を始め，2004年4月から24カ月齢以上の死亡牛全頭検査を行っている。と畜牛や死亡牛(24カ月以上)の全頭BSE検査，法的な生産から小売りまでの全流通過程のトレーサビリティ制度などは，欧州でも行われていない日本独自の制度である。

（2）日本のBSE疫学[2,3,4)]

わが国へBSEが侵入した可能性は以下の3つ(汚染牛，汚染肉骨粉，汚染獣脂)である。生体牛はイギリスからリスクの高かった時期に3回(5頭，9頭，19頭が関東，関東，九州)輸入され，それぞれの地域でレンダリングされ飼料に利用された。ドイツからは16頭が北海道に輸入された。アメリカ，カナダからは2003年にBSE牛が両国で発見されるまで，毎年乳牛数百頭が輸入された。しかし，輸入牛の汚染の可能性，年齢と廃用までの期間を考慮すると，ドイツ，アメリカ，カナダから輸入した牛がBSEに汚染していて流行の原因となった可能性はきわめて低い。

肉骨粉は1987年から，断続的・大量にイタリアから輸入されている(総量55,930 t)。デンマークからは1999〜2001年の法的肉骨粉輸入禁止まで30,500 t輸入されたが，この肉骨粉は感染価を低減させる新製法によるものであった。ドイツ，ロシアから少量の輸入，香港からイギリスの肉骨粉が迂回して輸入された可能性も否定できない(1994年は240 t)。しかし，いずれのケースも直接，北海道にこれらの肉骨粉が輸入されたという記録はない。動物性油脂の輸入によるBSE病原体の国内導入の可能性は1994〜2000年の間に関東と九州へ輸入されたオランダ産の動物性油脂(粉末油脂)1,245 tと，1989年に輸入されたスイス産(輸入港は不明)の動物性油脂22 tである。

これまでのBSE検査データから流行パターンの特徴は以下のように考えられる。

1）ヨーロッパの高汚染国に比べ汚染規模が比較的小さい。

2）ヨーロッパと同様，乳牛を中心に汚染が進んだ。
3）地域的，経時的にみて，不連続で散発的流行という形をとった。北海道以外では国内増幅は起こらなかった可能性が高い。
4）1996年後半～1998年生まれまで汚染がないことから，この間海外からのBSEの侵入はなかったと考えられる。1996年の関東および1999年の熊本生まれの陽性例はイギリス産の輸入牛，イタリア産の輸入肉骨粉がそれぞれ原因となった可能性がある。しかし，北海道の陽性例では，この時期(1995，1996年)，北海道に直接汚染を引き起こした海外からのリスク因子(イギリス・欧州の成体牛，輸入肉骨粉など)の侵入は，代用乳(オランダ産動物性油脂)を除くと，ほかの可能性はきわめて低い。

動物性油脂に含まれる不溶性不純物中のタンパクは少量であるが，オランダでは当時，脳・脊髄等の組織もレンダリングの原料として用いられていたこと，レンダリングによる不活化効果が低いこと，動物性油脂の利用がほぼ牛に限られていたこと，代用乳の対象となる幼牛では腸管でのタンパク透過性が高いことを考慮すると，当該代用乳のリスクは無視できない。北海道の1999～2001年生まれの陽性群は，1996年生まれの群のプリオンがレンダリングにより国内曝露と増幅を起こしたと考えられる。

3．食品安全委員会のリスク評価

（1）中間とりまとめ(2004年9月)[5)]

わが国のBSE対策の評価を行い「日本における牛海綿状脳症(BSE)対策について：中間とりまとめ」を公表した。日本のBSE浸潤率がどのくらいか？ とられた施策の効果は？ 日本でvCJD患者が出るリスクはあるか？ といった疑問に対し，科学的立場で公的に分析した最初の試みである。専門委員会はBSEに関する科学的不確実性を念頭に置きながら，科学的に判っていること，不明なことをひとつずつ明らかにし，これまでに得られた知見を整理した。またイギリスのデータを基にヒトへのBSE感染リスクを見積もり，日本のvCJDのリスク評価を試みた。これまでのリスク管理措置の実施状況を検証し，リスク低減効果を評価した。

結果として，日本におけるヒトのBSE曝露リスクは2001年10月の法規制後は無視できるレベルで，法規制前に食物連鎖に入ったBSE感染牛は5～35頭，vCJDの発生する可能性は，イギリスで予想されるvCJDの発生数(ワーストシナリオで4,000人)を考慮すると0.1～0.9人であると結論した。「中間とりまとめ」は多くのメッセージを伝えている。

1）BSEに関しては科学的に不確実性が多く現時点ですべてが説明できるわけではない。
2）発症牛あるいは感染末期牛ではSRMに異常プリオンタンパクの99%以上が集中しているが，と畜場で常にSRM除去が完全に行われていると考えるのは現実的でない。SRM以外に異常プリオンタンパクが蓄積する組織がないかどうかは現時点で判断できない等，SRM除去の限界を指摘した。
3）と畜場でのBSE全頭検査についても検出限界以下の感染牛があり得ること，全頭検査の結果から21カ月齢以上の牛では現在の検査法で感染が検出される可能性はあるが，それ以下の若齢牛でのプリオンの蓄積量は非常に少なく，20カ月齢以下の牛では陽性牛が検出されなかったという迅速検査の限界を示唆した。

（2）国内対策の見直し(2005年5月)[6)]

リスク管理機関から国内対策見直しの諮問を受け，BSE検査で検出困難な月齢の牛を検査対象から外したときのリスクの変動を評価した。イギリスの自然発症年齢の分布の評価，イギリスの実験感染例の評価，感染価の考え方，日本のBSE検査データの評価，イギリス・EU諸国の飼料規制の効果の評価，日本でのと畜や解体工程そして飼料規制の検証，日本での飼料規制等のリスク回避効果などを分析した。生体牛，食肉のリスク評価項目を設定し，定性的評価と定量的評価の2つの方法を試みた。

1）生体牛リスクでは1996年の肉骨粉使用規制が始まるまでにBSEの汚染は進行したと考えられる。このときの汚染は年間で最大43頭と推定した。その後規制通知が出され発生頭数は減少したと考えられる(欧州の規制効果から外挿すると2001

年末には推定年間３～14頭が感染)。対象となる20カ月齢以下の牛が生まれた2003年以後には汚染はさらに減少し，また年間のと畜頭数に対する20カ月齢以下の割合は1/5以下であるから，多くても感染牛は年間0.4～1.7頭と推定された。20カ月齢以下の感染牛のプリオン蓄積量はBSE検査で検出限界～検出限界以下(門部では，感染末期牛のプリオン蓄積量の約1/500以下）である。

2）食肉の安全性に関しては2001年までBSE対策はまったくとられなかったが，2001年以降はSRM除去，脊髄吸引，背割り後洗浄などの措置がとられた。評価を行った2005年のと畜場では衛生管理規範が実施されている。最悪のシナリオでも脊髄片の残存確率は0.2，枝肉洗浄後の最終汚染確率はさらに1/10となり0.02と考えられる(感染牛で汚染される確率は50年で１頭)。汚染量は検出限界程度(1/500以下)で，SRM除去(99.5%除去)により，さらに一層減少する。

生体牛リスクと食肉リスクを組み合わせた総合評価では，20カ月齢以下の個体を検査しても，しなくてもヒトの健康危害に関するリスクの差は非常に少ないという結果になり，全頭検査の限界を明示した。しかし，リスク管理機関は全頭検査を続ける方針を示したまま，リスク評価を求めるという矛盾を犯した。研究者も全頭検査の科学的意義と安全管理措置の違いを明確にできなかった。リスク評価と消費者の安心感の乖離も著しかった。科学的評価がリスク管理機関，消費者に理解されなかった事例となった。

３年間の検査ではリスク評価どおり，若齢牛での陽性例は摘発されなかった。しかし，３年後の2008年８月を迎えて，法的には若齢牛の検査は廃止されたが，すべての地方自治体は独自に若齢牛の全頭検査を継続することとした。リスク評価とリスク管理の乖離は継続することとなった。

(3) アメリカ・カナダの輸入肉等のリスク評価(2005年12月)[7)]

アメリカ・カナダの輸出プログラム(EVプログラム)で管理された牛肉・内臓を摂取する場合と，わが国の牛肉・内臓を摂取する場合のリスクの科学的同等性に関して諮問された。

生体牛リスク：輸入生体牛に由来するリスクはアメリカでは日本の約1.5～７倍，カナダは約４～６倍。肉骨粉の輸入リスクはアメリカでは日本の約1/12～1/47，カナダは約1/5,100。動物性油脂に由来するリスクはアメリカでは日本の約1/2，カナダは約1/12。しかし，BSEの曝露・増幅の最も大きなリスクはSRMの利用と飼料規制の不徹底である。アメリカ・カナダは現在もSRMを利用しており，飼料の交差汚染が完全には防止されず一定の割合で交差汚染が起こる可能性が残る。現時点で20カ月齢以下と考えられる牛の汚染はアメリカ，カナダの方が日本より数倍高いと考えられる。他方，アメリカ・カナダの飼育規模を考慮し，BSEの汚染率で比較すると100万頭あたりの汚染頭数はカナダが日本と同程度，アメリカはやや少ない可能性が考えられる。

食肉等のリスク：生理学的成熟度(A40)の月齢判定では20カ月齢以上の牛が入る可能性はあるが，BSE感染牛が１頭入る確率は高く見積もっても20年に１回弱である。と畜前検査では異常牛が見逃される危険性があるが，20カ月齢以下の感染牛で神経異常を示す可能性はきわめて低い。アメリカ・カナダでは健康と畜牛についてBSE検査が行われていないため検査によるリスク回避は不可能であるが，20カ月齢以下では検査により発見される可能性は非常に低い。EVプログラムではすべての牛からSRM除去を行う。ピッシングによるリスクは日本の方が高い。衛生管理手順(SSOP，HACCP)による管理措置は日本とアメリカ・カナダで変わらない。食肉に関してはEVプログラムが遵守されれば，BSEプリオンによる汚染の可能性は非常に低いと考えられる。

日本とアメリカ・カナダのデータが質・量ともに異なること，EVプログラムの遵守という仮定を前提に評価しなければならなかったことから，「科学的同等性を評価することは困難である。他方，EVプログラムが遵守されると仮定した場合のアメリカ・カナダの牛に由来する食肉等と，わが国の食肉等とのリスクの差は非常に小さいと考えられる」と結論した。なお，輸入解禁に踏み切ったとしても遵守が十分でなく，ヒトへのリスクを否定することができない場合は輸入を停止する必要がある。また，安全性を確