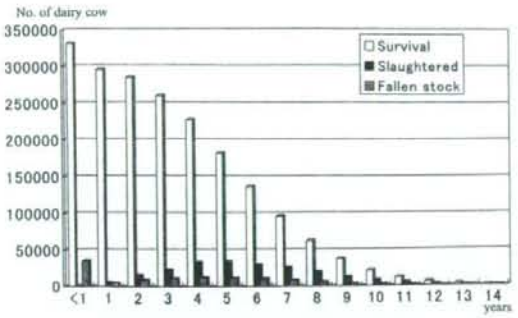


/ No. of cattle slaughtered (2005, data from the center for improvement of farm animals)
/ No. of dead cattle (2004, data from BSE surveillance of fallen stock)

Fig. 2. Survival curve of dairy cow in Japan.

2 EXPANSION AND TRANSMISSION OF BSE AND vCJD

Chronologically, the BSE outbreaks are separated into three categories in the world. They are in the UK, EU and other countries. BSE might be emerged in early 1980s in the UK, and first identified on 1986 and then confirmed in the general meeting of the OIE (Office de International Epizootics; World Animal Health Organization) on 1988. Meat and bone meal (MBM) including greaves was considered as a causative material by the epidemiological studies done in the UK [5] and the materials were banned to be used for animal feed as early as 1988. The specified risk material (SRM: SBO; specified bovine offal) was removed from the food chain in the end of 1989. During this time, live cattle and the extra MBM were exported from the UK to the EU, mostly. The feed ban was relatively effective, and BSE positive cases were peaked in 1992 and 1993, and gradually decreased later. However, in 1996, new variant Creutzfeldt Jacob Diseases (vCJD) were confirmed and considered to be derived from consumption of BSE infected meat (mainly MRM: machinery recovery meat), and it induced a world threatening panic. The UK government decided to start a measure of incineration of over thirty month cattle (OTM), and both exportation ban and real feed ban of MBM. From 1996 to 2000, 4.5 million cattle were burned and the UK succeeded to contain the BSE. In 2005, OTM regulation was relaxed to the BSE screening test in the slaughterhouse.

The EU countries recognized the risk of BSE and stopped importation of MBM from the UK on 1990, and as a result extra MBM of the UK was exported to Asia, America, and East European countries. The EU started a feed ban on 1994 and extra MBM was exported to other countries too. BSE contamination in the EU might be peaked during 1995 and 1996. In 2000, the EU started the active surveillance (rapid BSE test of cattle in the fallen stock and slaughterhouse), and introduced the real feed ban on 2001. In 2002, BSE positive cases detected by the active surveillance became a peak, and rapidly decreased later. In 2006, the EU accepted the deregulation of OTM of the UK, and started a re-importation of UK beef.

The third group, such as Japan, North America and East European countries etc., which imported live cattle, MBM or greaves from the UK and EU, were involved in BSE outbreak later than 2000 and detection of BSE cattle are still continuing now.

BSE positive cases reported in the World at 2007 January were as follows. The UK had more than 180,000 cases and the number is extremely high, and three EU countries, Ireland, Portugal, and France, detected 1,000 to 1,500 BSE cat-

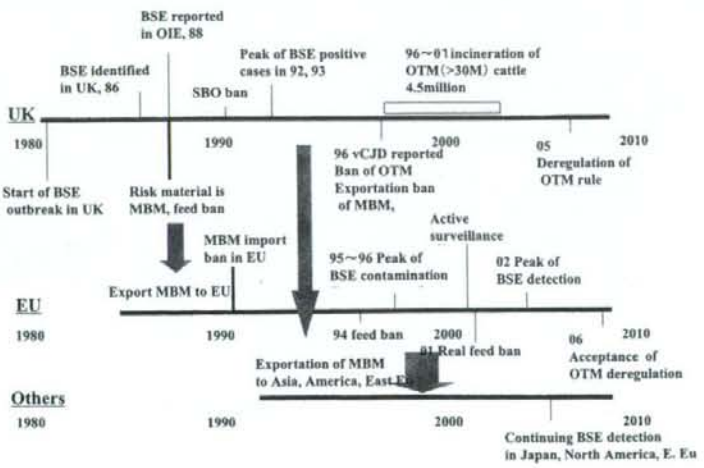


Fig. 3. Back ground of BSE outbreak in the world.

UK	184,453	Swiss	464	Poland	49	Canada	10	Liechtenstein	2
Ireland	1,578	Germany	404	Japan	31	Slovenia	7	Sweden	1
Portugal	996	Italy	134	Czech	24	Austria	5	Israel	1
France	976	Belgium	131	Slovakia	23	Luxemburg	3	Finland	1
Spain	654	Holland	80	Denmark	15	USA	2	Greek	1

UK	164	Swiss		Poland		Canada	1	Liechtenstein	
Ireland	4	Germany		Japan	1	Slovenia		Sweden	
Portugal	1	Italy	1	Czech		Austria		Israel	
France	21	Belgium		Slovakia		Luxemburg		Finland	
Spain	1	Holland	2	Denmark		USA	2	Greek Saudi Arabia	1

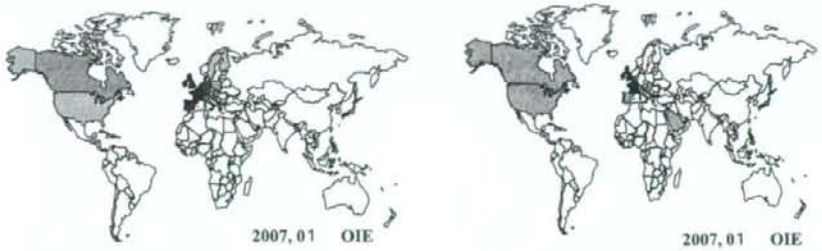


Fig. 4. BSE and vCJD cases in the world.

tle. Spain, Germany and Switzerland reported about 500 cases, and Italy, Belgium and Holland had about 100 cases. Japan reported 31 BSE cases, but now in October 2007, the number is 33. The total number of countries with BSE positive cases in the world is now 25.

Case reports of vCJD were a bit different from those of BSE. The UK had 164 patients at 2007 January, and they were relatively young (20 to 30 years old). In France, the number of BSE was not so high (about 1,000) when compared with the UK (180,000) and Ireland (1,600) or Portugal (1,000), but number of the vCJD patient was high, 21 cases, when compared with Ireland (4) and Portugal (1). Moreover, the age of patients is older (30 to 40 years old) than that of the vCJD in the UK. There are several possibilities on a high frequency of vCJD in France. That is a large quantity of contaminated meat product importation such as machinery recovery meat (MRM) from the UK, or dietary culture of the calf brain consumption. The total number of countries with vCJD patients in the world is now 11. From 2003, the vCJD entered into a new phase in the UK i.e., transmission of vCJD by blood transfusion was reported, and 4 cases were detected in the UK until now.

Thus, from 1986, it was cleared that BSE transmitted from cattle to cattle by MBM (including greaves) in the feed, and one full blown BSE cattle was considered to have an infectivity titer of 4,160 $CoID_{50}$ from the calf infection experiments by the EFSA (European Food Safety Authority) [1]. And complete feed ban, that is incineration of SRM, is the most effective measure. On the contrary, incomplete feed ban such as a ban of ruminant MBM to supply to ruminants, results in the cross contamination (cc). For example if one full blown BSE cattle, which has 4,160 $CoID_{50}$ infectivity was rendered by the ordinary methods (1 bar, about 60 min), the infectivity titer reduced to $\times 0.1$ (by rendering), and probable quantity of the cross contamination is $\times 0.005=2.1$ BSE cattle. It means one infected full blown BSE cattle might produce 2 positive cases by rendering within a mean incubation period.

From 1996, vCJD cases transmitted by consumption of the SRM of BSE cattle were reported. The first epidemics, in which the people with the prion gene of the codon 129 with Met/Met homozygote were involved, was a peak in 2000 to 2001 and then the number reduced. The SRM elimination from food chain is a principle countermeasure, and an elimination of BSE cattle which were born before the real feed ban by the BSE test in slaughterhouse is also effective.

From 2003, transmission of vCJD by blood transfusion was reported of 4 cases in the UK. In the case of BSE in cattle, the prion is restricted to the central nervous system or neuronal tissues, except for distal ileum. But, in the case of vCJD, the prion may be exist not only nervous tissues but also lymphoid tissues and blood circulation. By the Fisher's Exact Test comparing rate of infection after transfusion from vCJD (4 cases vs. 14) and CJD donors (zero vs. 116) suggests a statistically significance of the association of the value in two groups (<1% likelihood). It suggested that vCJD is more easily transmitted than ordinal CJD, which had been known to cause the iatrogenic infection.

3 LIST OF BSE POSITIVE CASES IN JAPAN

The total number of all aged cattle examined by the BSE test in the slaughterhouse from 2001 October to 2007 October is 7,446,809 heads and 20 BSE positive cases are reported. They are 2, 4, 3, 3, 5, 3 and 1 case from 2001 to 2007 fiscal year, respectively. On the other hand, sum of fallen stocks examined by the BSE test from 2003 April to 2007 July is 365,177 heads and 12 BSE positive cases are reported (the first case of BSE in Japan at 2001 September which was found in a farm, was excluded from this surveillance record). They are 1, 2, 3, 5, and 1 case from 2003 to 2007 fiscal year, respectively.

The 33 BSE positive cases reported in Japan until 2007 October were divided into 6 groups by a temporal and spatial difference, and BSE types (typical or atypical).

The pre-A-group is one case of Japanese black, female beef cattle which was born in the Iki, a very small island of Nagasaki prefecture (Kyushu) in 1992 February. She was the atypical BSE and detected 169 months old of age. This case might be independent from the outbreak of other typical BSE groups in Japan. Recently, the prion of this case was confirmed to be transmissible to the knock-in mice with bovine prion gene (the mouse with prion gene knocked out was introduced of the bovine prion gene: Tg bov) [2].

The A-group consisted of 13 typical BSE cases, and the characteristics of this group is as follows; they might be involved in a concentrated BSE contamination for a very short term and all the dairy cow (Holstein species) were born before or just after the administrative guidance for feed ban in 1996 April. The 10 cases in Hokkaido were born from 1996 February to August, and 3 cases in Kanto were born from 1995 December to 1996 March.

The B-group is one case of Holstein dairy cow which was born 1999 July in Kumamoto prefecture (Kyushu). She was the typical BSE case and no direct relation was considered with Pre-A-group cattle born in Nagasaki prefecture (Kyushu). British dairy cow and Italian MBM were imported to Kyushu area and it may be a cause of this case.

The C-group is now going to be a peak of BSE detection age. Until now, 16 cases were reported. The characteristics of this group are the all cattle were typical BSE and born exclusively in Hokkaido during 1999 August to 2001 August, before when the real feed ban was started (2001 October). The 15 cases were Holstein dairy cow and one case was female beef cattle of the Japanese black which was reared with dairy cow in the same farm by a compound management. Thus, she consumed the same milkreplacer, calf starter and the mixed feed as the dairy cow.

The D-group consisted of 2 male young cattle of Holstein species and both animals were born just after the real feed ban on 2001 October. One was born at 2001 October in Tochigi prefecture (Kanto) and became BSE test positive on 23 months old of age. He was diagnosed as an atypical BSE case. The other animal was born at 2002 January in Hyogo prefecture (Kansai) and became positive on 21 months old of age. He was belonged to the typical BSE case. In both cases, accumulation of the prion in the obex was very little and their prion were tried to transmit to the Tg-bov for two generations by the blind passages but the results were negative [4].

The Post-D group including all cattle which were born after 2002 February, there were no BSE positive cases until 2007 October.

Group (n)	Birth date	Birth place	Race	Characteristics
Pre-A (1)	1992 Feb	Nagasaki Iki island	Japanese black ♀	·Aged (169 M), Atypical case ·No relation with A group
A (13)	1995 Dec~ 1996 Aug	Hokkaido Kanto	Holstein ♀	·1996 Feb to Aug in Hokkaido (10cases) ·1995 Dec to March in Kanto (3cases) ·All BSE cattle were born before or just after the administrative guidance for feed ban in 1996 April
B (1)	1999 July	Kumamoto	Holstein ♀	·Only one case ·No relation with the Nagasaki pre-A group ·No direct relation with Hokkaido C group?
C (16)	1999 Aug ~ 2001 Aug	Hokkaido	Holstein ♀ Japanese black ♀	·Holstein 15 cases ·Japanese black 1 case (Dairy cow and beef cattle were reared in the same farm) ·All cattle were born before real feed ban on 2001 October
D (2)	2001 Oct 2002 Jan	Tochigi Hyogo	Holstein ♂ Holstein ♂	·Juvenile (23 M), Atypical case ·Juvenile (21 M) ·They were born just after real feed ban on 2001 October
Post-D (0)	After 2002			

Fig. 5. Grouping of BSE positive cattle.

4 BSE SAFE GUARD POLICY AND COUNTERMEASURES OF BSE IN JAPAN

After the first case of BSE was detected in Chiba prefecture (Kanto), consumers were led into a very big panic. The reason is that they lost trusts to the government and others as follows. There was distrust to the government because MAFF (Ministry of Agriculture Forest and Fishery) made a misunderstanding of the risk of BSE invasion, a lacking crisis measures, and no risk communication. Consumer gave a strong claim to the cattle breeder too, because they used MBM as cannibalism for cattle, and gave a priority to the economy rather than food safety. At the same time, the meat importer and processor conducted false applications for the compensation buying of the domestic beef by the MAFF, and false tags of meat in the course of the distribution in beef markets were also disclosed. Every day TV and newspapers mediated noisy but less signal information (low SN ratio). And finally, consumers distrusted the scientist who denied ZERO-risk and showed a scientific uncertainty, because majority of people believed that science is almighty, can explain everything and ensure safety. Thus, the consumers had an anxiety when they faced a scientific uncertainty.

Japanese government's policy of safe guard for BSE was consisted of three elements. The first is the SRM measures. The SRM of all aged cattle including the brain, trigeminus ganglia, spinal cord, vertebral column containing dorsal root ganglia, distal ileum, and tonsil/lingual tonsil are removed from food chain and incinerated. Besides SRM, other parts which are not used for human-consumption are rendered into MBM and incinerated, too. Thus, complete feed ban was performed. The second is BSE test which purposed surveillance of BSE on all fallen stock >24 month old and screening of BSE cattle in the slaughterhouse >21 month old. The third is traceability system. All cattle are identified by ear tag number with 10 digits and registered in the national control center in production level. A small portion of the all cattle meat from carcasses was stocked for the DNA diagnosis and 10 digits were displayed in the market or meat restaurants. This is the beef traceability from the slaughterhouse to the markets in the distribution level. The 10 digit can be traced by a personal phone or internet.

Another government policy introduced after BSE outbreak in Japan is separation of risk assessment and management; that is an introduction of risk analysis system on food safety. Risk analysis consists of three elements, i.e., risk assessment, risk management and risk communication. In Japan, risk assessment is done neutrally and scientifically in Food Safety Commission (FSC) of the Cabinet Office. The purpose is scientific assessment of hazards for human health. On the other hand, risk management is conducted politically by MAFF or MHLW (Ministry of Health Labor and Welfare). The purpose is making a standard or regulation based on the risk assessment with considering risk benefit or cost benefit. Risk communication is an exchange of information or opinion about the risk by all stake holders and it is performed by the public comments and risk communication meetings.

Although there occurred a nationwide panic among consumers after the first BSE case, the activities of three organizations (MAFF, MHLW, FSC) has been successful in obtaining confidence or trust of consumers relatively short time. As for the countermeasures, in 1996 the MAFF started BSE surveillance test of the risk animals, and the TSE including BSE was involved in the notification diseases in 1997. After the first case of BSE, complete feed ban for the use of MBM, removal of SRM and BSE test in the slaughterhouse had been obligated (2001 October). In addition, BSE-related risk is assessed by the Prion Expert Committee, Food Safety Commission, in the Cabinet Office since July 2003. Then, introduction of traceability system, and several BSE related risk assessments were conducted [3].

Basic strategy of the risk management for BSE in Japan is importation ban of MBM, live cattle and beef from the countries where BSE has been detected. Cattle are grown with the feed produced in the completely isolated facility from those producing pig or poultry feed in order to avoid cross contamination. In the farm, all cattle are identified by the traceability system and fallen stock in the farm over than 24 month old is BSE tested. In the slaughter house BSE test is conducted to over than 21 month old cattle by the law (cost of the BSE test for cattle younger than 20 months old, however, is covered by the national budget during three years; from 2005 August to 2008 July) and the positive cattle is incinerated. The SRM is removed from all cattle regardless of the result of BSE test and incinerated. Both the slaughterhouse and meat processing facilities are completely separated between cattle and pig meat to avoid cross contamination. Non human-consumption materials from cattle are rendered and finally incinerated.

Thus, containment of the BSE risk is based on stopping cattle to cattle, cattle to human and human to human infections. These are MBM feed ban, control of feed production and compost regulation (cattle to cattle). BSE test and SRM removal in the slaughterhouse, and regulation for drug production using BSE free cattle materials are safeguards for cattle to human infection. Ban of blood transfusion and organ transplantation from the high risk person who stayed in the UK until 1966 or EU, is precautious to prevent human to human infection. Japan is one of the most strictly regulated countries in the measures of BSE, but we have already conducted deregulations of some measures, too. They are use of swine MBM for swine feed after complete separation of feed factory or feed producing facilities (2005) and use for fish feed (2007), after risk assessment of FSC. Liquid compost treated with high pH from cattle MBM (2004), and ash of cattle MBM or bone can be used for the compost (2005). And revision of BSE test in slaughterhouse for cattle more than 21 month old was settled in law (2005) by the assessment of FSC.

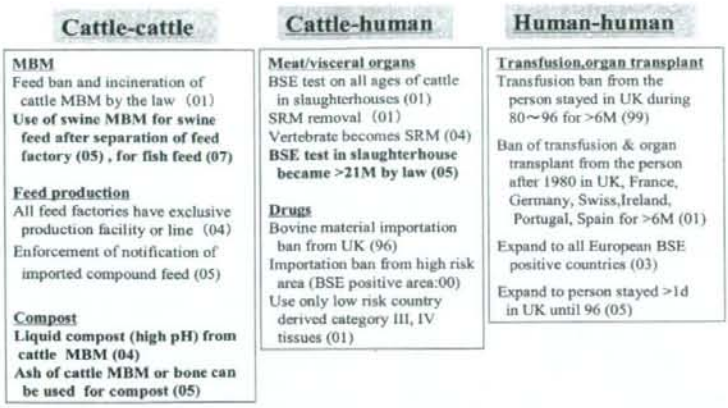


Fig. 6. BSE countermeasure in Japan.

5 INVASIVE BSE RISK SCENARIOS IN JAPAN

The main work of this study is to identify, as hypotheses, all feasible sources and routes of infection for the BSE cases discovered in Japan, and to study the probability of each hypothesis. Unlike ordinary microbial infections, BSE has an extremely long incubation period for several years (mean incubation period is Ca 5 ± 1.5 years). It is technically impossible to detect genome of pathogen or antibody, and there are extremely few confirmed cases. Nevertheless these difficulties, it is hoped that these research results will be of use in preventing future outbreaks, which is the primary object of the epidemiology.

The principle and strategy of this epidemiological study of BSE in Japan are as follows. 1) BSE risk status in Japan is divided into 3 stages, i.e., before 1996 April when administrative guidance for feed ban of cattle MBM introduced. After that to 2001 September, the first case of BSE in Japan, then, after 2001 October with real feed ban in the law. 2) Make hypotheses depending on the invasive risk scenarios and propagation risk of BSE in Japan, and they are checked by evidences, case control study or statistics. 3) Grouping of BSE cattle was conducted time sequentially and spatially; that is Group-A (1995, 1996, born in Hokkaido, Kanto), Group-B (1999 born in Kyushu), Group-C (1999–2001 born in Hokkaido), Group-D (young cattle born after real feed ban) and Pre A- , Post-D groups as described above.

The risk of the BSE agent being introduced to Japan through imports of the live cattle can be divided into four scenarios. These are the 5 cattle born in southern England and imported into the Kanto region in 1982, the 9 cattle born in southern England and imported into the Kanto in 1987, the 19 cattle born in southern England and imported to Kyushu in 1988, and the 16 cattle born in Germany and imported to Hokkaido in 1993. All history of each animal in Japan is cleared. On studying the respective import lots, the origin of live cattle imports from the UK (all were dairy cow) gradually shifted from central to southern England, where BSE contamination was more intense. This study also clarifies issues such as where the cattle were reared in Japan after import, and the corresponding slaughterhouse processing after exhaustion of the reproductive cycle of them, MBM manufacturing processes, and where the cattle were re-used as MBM. In Japan, about 160 slaughterhouse and rendering factories, as well as feed factories (about 140) are relatively regional industries. Almost all animal feed were produced and consumed in local areas, for example Hokkaido feed products are 100% consumed in Hokkaido.

The risk of imported MBM is divided into three scenarios, i.e. imports from Italy, Hong Kong and Denmark. The scenarios for MBM imports from Germany or Russia were not considered, since the risk from these is thought to be extremely low. This is because the respective import volumes were small and there was hardly any manifestation of BSE among cattle produced in those countries at the time of import. In the case of Italy, 55,930 tons of Italian MBM had been imported between 1987 and 2001. Moreover, MBM imports from Italy involve a number of factors, including the state of BSE incidence in Italy, variations in the volume of MBM imported, and changes in the MBM manufacturing processes. Namely, the 656 tons imported between 1987 and 1993 (no imports in 1991 and 1992) to Yokohama (Kanto), Nagoya (Chubu) and Moji (Kyushu) were categorized as M1 (high risk), the 5,408 tons imported between 1995 and 1998 to Nagoya (Chubu), Yokohama (Kanto) , Kobe (Kansai) and Kagoshima (Kyushu), of which the 4,802 tons imported after June 1998 were heat-treated at 133°C, 3 bar, 20 min as M2 (moderate risk), and the 49,846 tons imported between 1999 and 2001 to West Japan (Kyushu, Kansai, Chubu) as M3 (low risk). In the case of Denmark (BSE positive cases are only 15 on 2007 January), 30,500 tons of Danish MBM, which were processed by 133°C, 3 bar for 20 min, had been imported in 1999–2000 (low risk).

The risk of importation of animal fat and tallow is divided

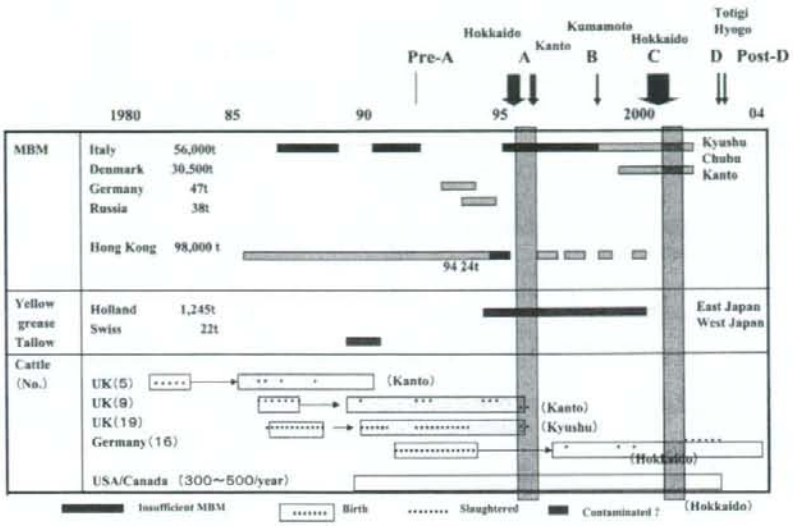


Fig. 7. List of invasive risks of BSE in Japan.

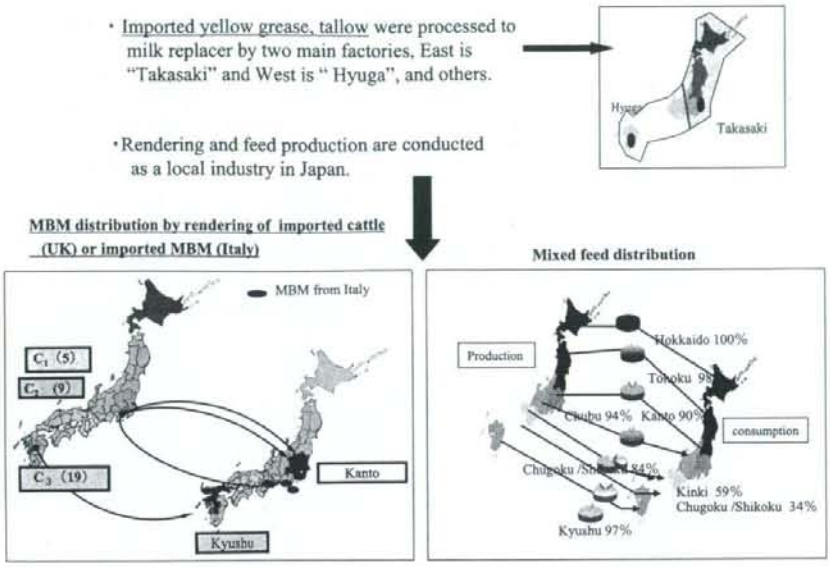
into two scenarios. These are the 1,245 tons of Holland animal fat (powdered fat) imported into Kanto and Kyushu between 1994 and 2000, and the 22 tons of Switzerland animal fat (tallow) imported in 1989. Imported animal fats were used for producing milkreplacer. In Japan, there are a few milkreplacer factories and they shared broad area such as East of Japan or West of Japan.

The BSE risk status of Japan was classified tentatively into three periods as follows by the BSE risk assessment. Before 1996 April, there were no regulations for rendering and animal feed production, and the invasive risk was high because importation of live cattle from the UK was moderate, Italian MBM was high, and animal fat from Holland was very low. The stability was extremely unstable, because the rendering condition was poor (about 110°C, 1 bar, 60 min) and SRM was used for the cattle feed. Thus BSE risk status of this period is GBRIII or undetermined. The period from 1996 April to 2001 September, invasive risk was moderate because live cattle importation from U.S.A. and Canada is low, MBM imports from Italy and Denmark was moderate and animal fat from Holland was very low. The stability was unstable because partial feed ban was introduced (1996 April, Administrative guidance for feed ban of cattle MBM) but SRM was still used for rendering and feed production was conducted with a condition of cross contamination. Moreover, MBM was used for a supplement in many dairy farms. Thus, BSE risk status of this period was GBRIII or undetermined. After 2001 October, the BSE risk status became low ~negligible, because invasive risk of live cattle from U.S.A. and Canada was low, complete ban of MBM importation was negligible risk and animal fat importation from Australia was negligible risk. The stability was very stable because SRM was incinerated. Moreover, the slaughterhouse, rendering facility and feed factory were separated between cattle and pig or chicken systems avoiding the cross contamination. Thus, BSE risk status of this period is GBRI or the controlled risk.

6 CHARACTERISTICS OF BSE OUTBREAK IN JAPAN

The feature of the BSE outbreak in Japan is a bit different from those of European countries as follows. 1), Epidemic size is relatively smaller than those in European countries. Extremely intensive BSE test had been conducted in Japan and a nationwide traceability system by the MAFF was established. Thus, precise data on each BSE positive case could be listed up, and all population of cattle including healthy slaughterhouse animals (from 2001 October) and farm fallen stocks (from April 2004) were BSE tested. 2), Un-even distribution of BSE cases was observed. Until now, Hokkaido is a core of BSE epidemic in Japan, and two different time outbreaks were occurred there. 3), Dairy cow are mainly involved. And 4), Sporadic, discontinuous outbreaks were occurred temporally-spatially. That is, in 33 BSE test positive cattle in Japan, the birthplaces of 26 cases are Hokkaido and other 7 animals are born in other prefectures. If the atypical cases were eliminated (case No. 8 and 24) from the outbreak of typical BSE, 26 in 31 cases (84%) were born in Hokkaido.

Another characteristic of relatively large sized BSE outbreaks in Hokkaido is a difference of the patterns of the birth dates among cattle of the Group-A and -C. Mean and standard deviation of the A-group cattle birth date interval are 3.6 ± 1.8 months. It is concentrated in an extremely narrow period. However, those of the C-group are 10.5 ± 6.6 months until now. By the statistical analysis, it can be con-



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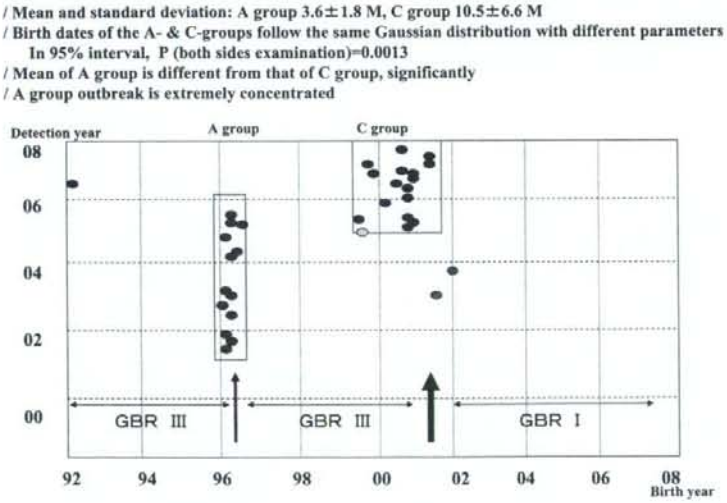
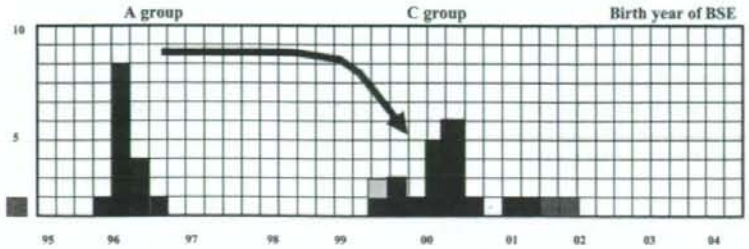


Fig. 9. Features of BSE outbreaks in Hokkaido.

sidered that the birth dates of the group-A and -C follow the same Gaussian distribution with different parameters. In the 95% confidence interval, P value (both sides examination) is 0.0013. It suggested that the cause of A-group might be different from that of C-group significantly, and possible contamination of A-group might be single or a few number of lots including the causative agents (BSE prion).

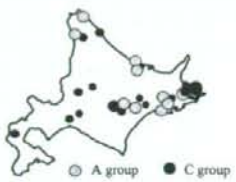
Spatial distribution of BSE cases in A-group Hokkaido was closely related to the milkreplacer- and feed-distribution route by the Kushiro-route. Another different characteristic between A- and C-groups is spatial husbandry background in Hokkaido. The stock raising of BSE positive area in Hokkaido was different, that is in the A-group, only beef cattle and dairy cow are reared in the areas of 9 out of



- **The former half in 1996, concentrated contamination of BSE was occurred in Hokkaido (A group), then there was no BSE invasion, and indigenous propagation resulted in the group C (1999 to 2001)**
- **Outbreak of BSE after 2002 might be controlled (assessment can be done within several years)**
- **If BSE case born after 2002 is not detected, then Japan becomes the negligible risk country in 2013**

- **Feed route in Hokkaido**

A group contamination via Kushiro



C group in the whole Hokkaido (via Kushiro & Tomakomai)

Fig. 10. Chronological and spatial prevalence of BSE.

Prevalence of BSE in A-group; 10 positive/ 75,000 heads (P=1:7500(population))
/ If Pre-A group is identical to A-group, sample size to be needed for detection of BSE case in Pre-A-group is
/ Sapling size $n=\{1-(1-P)^{1/d}\}\{N-d/2\}+1$
N: population size, d: BSE case in population, P: probability to detect 1 positive case at least in the sample
/95% intervals; sample size of 1 BSE positive case is included (population is more than 10,000 heads to infinitive)
n=7810~7980 heads (about 7,900 heads are BSE tested, 1 case becomes positive (95% confidence intervals))
/Actuary, no positive case in 56,000 heads (95% confidence, 6.6 cases become positive in the population size)

/ Examination of significance of the association of the value in the Group-A and Pre-A group
/ 10 cases positive, but 75,000 negative in the A-group
/ No positive case and 56,000 negative in the Pre-A group
By χ^2 examination
$\chi^2 = (|ad-bc|-N/2)^2 xN / (a+b)(c+d)(a+d)(b+d) = (560000-65500)^2 x131000 / 131000x10x56000x75000$
$=5.95$, χ^2 :0.05 (3.84), 0.01(6.63) 95% confidence intervals: significant difference between two groups

If Fisher's exact test is used instead of χ^2 test, because of the cell including an expectation value being less than 5.
/ The cell includes zero, $P=n_1C_a x n_2C_c / {}_NC_{m1} = n_1!n_2!m_1!m_2! / N!a!b!c!d!$
$=75010!56000!10!131000! / 131010!10!75000!0!56000! = 0.00378$
/P x 2 =0.00756 is smaller than 0.01, thus less than 1% likelihood, null hypothesis is rejected.

Fig. 11. Statistical analysis.

10 BSE positive areas (pig was reared in one area, 1 out of 10 BSE cases), but in the C-group, pig and/or chicken were reared 5 out of 11 areas (8 out of 15 BSE positive cases). It suggest that the cause of contamination of the A-group might be closely correlated with only cattle, but that of C-group with pig and/or chicken breeding. Thus, Group-A contamination might be milk replacer or supplement, which is specifically used for cattle, and Group-C contamination might be cross contamination of cattle feed in the factories and/or milk replacer.

Population study on the Hokkaido dairy cows suggested followings. About 160,000 to 170,000 dairy cows are born every year in Hokkaido (half number of Japanese dairy cow) and majority of the population are slaughtered from 4 to 7 years old of age (total ~65,000 head per year). Mortality rate of the fallen stock is high during 2 to 7 years old of age (total ~30,000 head per year).

By the statistical analysis, 1), The Before-group-A (Pre-A

group, dairy cows in Hokkaido which were born before 1995 August, and there was no positive case in the BSE test; zero/56,000 tested) and Group-A (they were born from 1995 Sept. to 1996 August, and by the test, BSE positive cases were detected; 10/75,000 tested) are different on the BSE prevalence. It suggested that BSE contamination occurred suddenly at the latter half of 1995 in Hokkaido.

2), Statistically Group-A and Post-group-A (they were born from 1996 Sept. to 1999 July, and by the test, zero/240,000 tested) are different on BSE prevalence by statistical analysis. It suggested that during 1997 and 1998 there were neither BSE invasion nor propagation in Hokkaido.

3), Statistically Post-group-A and Group-C (they were born from 1999 August to 2001 October, and by the test, BSE positive cases were detected; 15/143,000 tested) are different on BSE prevalence. It suggested that Group-C outbreak might be induced by BSE propagation in Hokkaido (from Group-A). It is because spatial overlapping of the positive cases in Groups-A and -C was observed, no BSE case was detected in the cattle born during Post-A period (1997 and 1998), and the interval between Group-A and -C might be consistent with the BSE incubation period. Other than Hokkaido (in Kanto and Kyushu), indigenous propagation of BSE was not detected by the extensive nationwide BSE surveillance until now.

Strangely, however, it is very important to point out that the invasive BSE risk into Hokkaido was very poor. That is, all the UK imported live cattle were rendered in Saga prefecture (Kyushu) and Kanagawa prefecture (Kanto), and consumed as animal feed outside of Hokkaido (mainly in Kanto, and Kyushu). There were no reports of Italian MBM importation into Hokkaido (they were imported into Kanto, Chubu, and Kyushu). The Germany live cattle were rendered in Hokkaido, but it was occurred later than 1996 (after the A-group outbreak in Hokkaido) and the MBM used in the Hokkaido feed factory from 1995 April, was derived from Hokkaido cattle at that time. Thus it may be impossible that the MBM produced at that time (the latter half of 1995) was contaminated by BSE prion in Hokkaido. On the contrary, all BSE cattle of the A-group drunk the same milkreplacer made of Holland animal fat (Hokkaido 10 cases, and Kanto 3 cases).

7 BSE CONTAMINATION SCENARIO AND ITS CONTRADICTION

The hypothesis that animal fat imported from Holland from 1995 to 1996 being a causative agent, is examined by a case control study. The farms of Group-A (in Hokkaido and Kanto) and control cases (population control), which were randomly sampled as 200 farms from 20,000 dairy farms in the East of Japan where the milk replacer made from Holland animal fat might be used. The randomness of the selected farms was confirmed by statistics. As a result, the concerned milkreplacer shared about 30% in Hokkaido at that time. The case control study resulted in 1% significance on P<0.0001, odds ratio=39.3, and 95 % confidence intervals (4.9–312.9), suggesting that the hypothesis is statistically significant. The same results was obtained by the comparison of expectation value and actual value using milkreplacer of the corresponding factory (Pure milk / Milfood-A-Super made of Holland animal fat) and BSE positive case in Hokkaido. Whether the A-group being independent or not was examined (share of the milkreplacer was in Hokkaido=30 %), and it was statistically significant (p=0.00001).

When compared feeding history of the Group-A and Group-C cattle, the factory of the milk replacer for Group-A was common, and other feed such as calf starter and mixed feed were not produced in the same factory. In the case of Group-C, not only milk replacer but also calf starter and mixed feed were produced by various factories.

/ Case control study. Group A Hokkaido, Kanto and control farms (population control) randomly sampled 200 farms from 20,000 dairy farms in East Japan and the randomness was confirmed by statistics.

Case control study

	BSE	Normal	Total
Case (Takasaki)	12 (13)	36	48 (49)
Control (Others)	1 (0)	118	119 (118)
Total	13	154	167

1% significance P<0.0001
Odds=39.3
95 % intervals (4.9-312.9)

/ Using milk replacer of "Takasaki" (Pure milk/ Milfood Asup) and BSE positive case in Hokkaido A group are independent or not (Share in Hokkaido=30 %).

Expectation value

	Takasaki	Others	Total
Positive	3	7	10
Normal	16497	38493	54990
Total	16500	38500	55000

Actual value (p=0.00001)

	Takasaki	Others	Total
Positive	10	0	10
Normal	16490	38500	54990
Total	16500	38500	55000

Fig. 12. Statistical analysis.

In deed, the record of animal fat importation from Holland (shipping days in 1995, 1996) confirmed that the 1st lot was1995 July 15, 2nd lot 1995 September 29, 3rd lot 1995 December 9, and 4th lot 1996 January 29, respectively. In the corresponding factory, the milkreplacer was produced from 1996 January and February (Pure milk, and Milfood-A -super) from the 2nd lot which was arrived at 1996 December, and transported to the East of Japan. The Hokkaido A-group dairy cow were born from 96 February to 96 August and in Kanto from 1995 December to 96 March. The maximum term of validity on the milkreplacer may be about 6 months after production. Thus, if 2nd lot was contaminated, all dairy cows might drink the same milkreplacer of this lot.

Thus, the circumstantial evidences suggested that the milkreplacer containing Holland animal fat might be the cause of A-group outbreak in Hokkaido and Kanto on 1995 and 1996. The Holland epidemiological study reported on the animal fat as follows. There were possibilities that fatty tissues surrounding intestine including the nervous tissues and the ileum which might be infected with BSE prion, were rendered at that time. There was possibility, when collecting the skull and spinal cord for rendering, the CNS was

case	1-2M (milk replacer)	3-6M (calf starter)	7-12M	>1y
1	**Milfood Asup**	Milfood B flake	Young cow lead , Infant green	Young cow lead
2	**Milfood Asup, Pure milk H**	Milfood B green	New sun lucky18	New sun lucky18
4	**Milfood Asup**	**Milfood Asup**	**Milfood Asup** New step 16	New step 16
6	**Milfood Asup**	Milfood B	Support 70	Support 70
7	Milk	**Milfood Asup** Infant green	Infant green	Young cow lead
11	**Milfood Asup**, Milfood B flake	Support 70	Support 70	Support 70
13	**Pure milk H**, Milfood B green Infant green	Infant green	Infant green New lead 18	New lead 18
15	**Milfood Asup**, New step 18, Calf top L	New step 18 Calf top L	New step 18	New step 18
16	**Milfood Asup**, Milfood B flake	Young cow lead	Young cow lead Tokuhai 18ME	Tokuhai 18ME
19	**Milfood Asup**	**Milfood Asup**	Calf meal ,Yodel 18	Yodel 18
3	**Pure milk**, Moret, premium	**Pure milk**, premium starte		
5	Milk, **Pure milk**	**Pure milk**	Maybypass40	Maybypass 40
10	**Pure milk**	**Pure milk**		

Fig. 13. Feed history of Group A cases (Hokkaido, Kanto).

Case	~2 Months	~3 Months	~5-8 Months	~12 Months
14	**Milfood A-Sup**, New calf lead 24	New calf lead 24	New calf lead 24	New calf lead 24
17	**Milk**, **Milfood A-Sup**, New lead 20	New lead 20	New lead 20	New lead 20
18	Holstarter F, Calf Succul, Sewer Succle	Holsterte F	Dairy Royal 18	Dairy Royal 18
20	**Milfood A-Sup**, Manna club	**Milfood A-Sup**, Manna club	New calf lead 24, New lead 16	New calf lead 24, New lead 16
21	**Saturaku new calf**, Dairy Royal 16	**Saturaku new calf**, Dairy Royal 16	Dairy Royal 16, Dairy Royal 18	Dairy Royal 18
22	**Milk**, **Milfood A-Sup**, Quality calf M	New step 18, Konsen 18	New step 18, Konsen 18	Konsen 18
23	**Milk**, **Milfood A-Sup**	Milfood B green	New calf green, New lead 18 Kamikawa 16	New calf green, New lead 18 Kamikawa 16
25	Calf top, New make star	New calf lead 24	New calf lead 24	New calf lead 24
26	**Milfood A-Sup**	**Milfood A-Sup**	Kaikyo H18	Kaikyo H18
27	**Milfood A-Sup**, Milfood B green	**Milfood A-Sup**, Milfood B green New calf green	New calf green, New calf lead 24	New calf lead 24
28	**Milk**, **Milfood A-Sup**, New calf green	New calf green	New calf green	New calf green
29	**Milfood A-Sup**	New calf green	New calf green, New calf lead 24	
30	**Milfood A-Sup**	Dairy star 18	New calf lead 24	
31	**Milfood A-Sup**	Calf starter, New calf green	New calf lead 24	
32	α MilkA, α Five star	Parity 18	Parity 18	Parity 18

Fig. 14. Feed history of Group-C cases in Hokkaido.

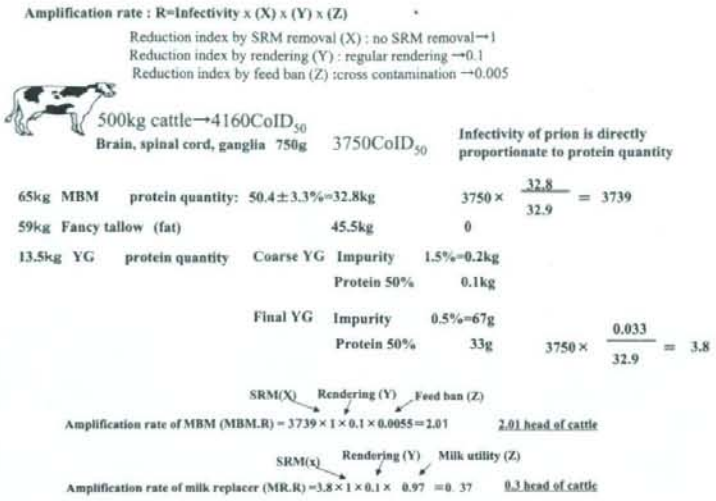


Fig. 15. Theoretica infectivity of milk replacer

included in them. It was sure that the SRM removal was not obligated before 1997 (first BSE case was detected) and nervous tissues might be included. Rendering fat should be ruled containing less than 0.15% impurities, however, before it was ruled as 0.5%. On the contrary, fancy tallow had less than 0.02% impurities.

If the hypothesis of Holland animal fat as causative material was accepted, there are several unexplainable points. By the EFSA evaluation, one full blown BSE cattle is considered to have an infectivity titer of 4160 (if only the nervous tissues was used for rendering, infectivity titer is 3750) $CoID_{50}$. When one adult cattle is rendered, resultant 65 kg MBM may contain 32.8 kg protein, and 13.5 kg yellow grease of 0.5% impurities may contain 33 g protein. Theoretical MBM infectivity by the ordinal rendering method (about 110°C, 1 bar, 60 min) with cross contamination on 1995 in Holland is 2.01 $CoID_{50}$ ($3739 \times 1 \times 0.1 \times 0.005 = 2.01$). On the other hand, theoretical animal fat infectivity at that time in Japan may be 0.37 $CoID_{50}$ ($3.8 \times 1 \times 0.1 \times 0.97 = 0.37$). Therefore, the contaminated milkreplacer lot had to be made of 54~81 full blown BSE cattle theoretically in order to induce A-group outbreak (about 20 ~30 cases), provided the worst scenario was accepted (impurity of the animal fat was 0.5 %, including the central nervous system and other nervous tissues such as ganglia). By the Holland epidemiology, however, such kind of high dose of BSE contamination could not be occurred. Probably there are missed risk factors in this estimation, such as un-homogeneous protein impurity in the animal fat or higher content of SRM in animal fat occasionally, and high intestinal absorption of neonatal cattle (< 1 month old) than the calf which are used for the experimental infection (3~6 months old). Collection of scientific evidences relating these possibilities will be needed to clarify the true causative material.

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Short communication

Induction and sequencing of Rousette bat interferon α and β genes

Tsutomu Omatsu [a,*], Eun-Jung Bak [a], Yoshiyuki Ishii [a], Shigeru Kyuwa [a], Yukinobu Tohya [b], Hiroomi Akashi [b], Yasuhiro Yoshikawa [a]

[a] *Department of Biomedical Science, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan*

[b] *Department of Veterinary Microbiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan*

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Abstract

Bats are considered to be natural reservoirs for several viruses of clinical importance, including rabies virus, Nipah virus, and Hendra virus. Type I interferons (IFNs) is an important part of the immune system in the defense against viral infection. To investigate the function of type I IFNs upon viral infection in bats, the nucleic acid, and amino acid sequences of Egyptian Rousette (*Rousettus aegyptiacus*) IFN-α and -β were characterized. Sequence data indicated that bat IFN-α consists of 562-bp encoded 187-aa, and IFN-β consisted of 558-bp encoded 186-aa. Phylogenetic analysis of the overall identity of IFN-β shared the highest sequence homology with pig IFN-β in both nucleotide and amino acid level. Stimulation of bat primary kidney cells (BPKCs) and bat lung cell lines, Tb-1 Lu, with polyinosinic–polycytidylic acid (poly(I:C)) or exogenous bat type I IFNs resulted in increased type I IFNs mRNA expression in BPKCs, but not in Tb-1 Lu. Characterization of the bat IFN-α and -β genes allows understanding of the immune responses upon stimulation in different tissues, thus providing practical strategies for control and treatment of clinically important diseases. These results are important especially for the virus infection, and suggest that future molecular studies on virus infection experiment of bats *in vitro* will require careful consideration of the differences of type I IFN expression patterns in different cell types.

Keywords: Bat; Type I interferons; Bat kidney primary cells; Tb-1 Lu

1. Introduction

Bats, Chiropteras, are well-known vectors of rabies and some studies indicate that they may also naturally harbor some emerging viruses such as Nipah virus, Hendra virus, bat-SARS-CoV and Ebola virus (Chua et al., 2002; Halpin et al., 2000; Lau et al., 2005; Leroy et al., 2005; Mayen, 2003; McColl et al., 2000; Normile, 2005). Bat has two suborders, Megachiroptera (flying fox) and Microchiroptera (insectivorous bat). Many emerging or re-emerging viruses, such as rabies, Nipah virus, and Hendra virus, were isolated form Megachiroptera. In particular, European bat lyssavirus type I was also isolated from *Rousettus* sp. (Van der Poel et al., 2000; Wellenberg et al., 2002; Wong et al., 2007). Bats were thought to have an important role for the infection cycle of these emerging and re-emerging viruses. *In vivo* experiment,

* Corresponding author at: Laboratory of Vector-borne Viruses, Department of Virology I, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan.
Tel.: +81 3 5285 1111; fax: +81 3 5285 1208.
E-mail address: t-omatsu@nih.go.jp (T. Omatsu).

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Ebola virus inoculation studies showed that both flying foxes and insectivorous bats support viral replication and circulation with high viral titers without becoming ill (Swanepoel et al., 1996). Studies *in vitro* have shown that Ebola virus VP35 protein blocks the activation of interferon regulatory factor 3 (IRF-3) and Ebola virus VP24 protein inhibits interferon (IFN) signaling (Basler et al., 2003; Reid et al., 2006). These data suggested that Ebola virus might evade the anti-viral activity of IFNs in bat cells. Therefore, it is crucial to investigate IFN regulation and function in bats because few immunological studies have been reported for this animal species.

Cells have many responses to viral infection. One of the responses is the secretion of type I IFNs which are composed of multiple α subtypes and a single β subtype (Sen, 2001). Type I IFNs expression utilize two signal transduction pathways; the Toll-like receptor (TLR)-dependent pathway and TLR-independent pathway. In TLR-dependent pathway, cells recognize viral double-strand RNA, single-strand RNA and CpG DNA via TLR, and subsequently IFN-β is induced. In TLR-independent pathway, intracellular sensors such as retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene-5 (mda-5) detect viral components in the cytoplasm, and transactivate IFN-β mRNA (Hiscott et al., 2006). Expressed IFN-β binds to the type I IFN receptors and activates numerous IFN-stimulated genes, such as the protein kinase R (PKR) gene, the 2′–5′ oligoa-denylate synthetases (OAS) gene, and the myxovirus resistance (Mx) gene. The product of these genes controls viral infection (Samuel, 2001). Viral double-stranded RNA (dsRNA), a viral intermediate in the proliferation of many RNA viruses, is known as an IFN-inducer through these sensors (Gitlin et al., 2006). Polyinosinic–polycytidylic acid (poly(I:C)) is a synthetic mimetic of viral dsRNA and a strong inducer of type I IFNs *in vivo* and *in vitro* via these sensors (Hertzog et al., 2003).

Type I IFNs stimulate anti-viral activity as mentioned above; however, such studies in bat have not been possible because the bat IFN related genes had not been previously identified. In this study, we determined the sequence of a subtype of IFN-α and IFN-β from *Rousettus aegyptiacus*, including the full open reading frames (ORFs), and analyzed phylogenetically based on IFNs from other mammals. In addition, the up-regulation of these mRNAs in both bat primary kidney cells (BPKCs) and a bat lung cell line, Tb-1 Lu was examined using poly(I:C) or bat type I IFNs derived from BPKCs.

2. Materials and methods

2.1. Preparation of cDNA from bat genomic DNA

Fresh liver sample and whole blood of *R. aegyptiacus* under anesthesia with ketamine (5 mg/ml/kg) and medetomidine (0.2 mg/ml/kg) were collected by heart puncture. Bat liver was fixed with 10% neutral buffer formalin. Bat genomic DNA was isolated from fixed liver with the Wizard Genomic DNA Purification kit (Promega, Madison, WI) and stored at −20 °C until usage.

2.2. Sequencing of bat IFN genes

Bat genomic DNA sample was used as a template of polymerase chain reaction (PCR) using TaKaRa Ex Taq (Takara Bio, Ohtsu, Shiga, Japan). Forward and reverse primers of IFN-α and IFN-β for PCR were designed from the sequence data of human, mouse, cat, pig, and horse IFN-α and IFN-β (Table 1). The accession numbers of these data in GenBank are as follows: IFN-α

Table 1
Sequence of each PCR primers

Primer name		Sequence (5′–3′)
IFN-α	Forward	CTC TCT AGG ATG TGA CCT GCC TCA GA
	Reverse	ACA GGG GCT GTG TTT CTT CTC
IFN-β	Forward	GCT TGG ATT CCA ACT AAG AAG CAG C
	Reverse	ACA GAC GCT GTA CTC CTT GGC CTT CA
GAPDH	F	GAT GGA GCA TCA TAC TGA TCC
	R	GAC CTT CTA CCA CTA CCC AAA
IFN-α	F3	ACA GAG GCA GGT CTT CAC AAC CTA GA
	R2	GAG AAG CAT TTC CAT GTT GAA CCA G
IFN-β	cdsF	TAG GTG ATA GTA GGC ACC ACT GTT CC
	cdsR	CTT TCT CAG AAG TAC AGG CGG AGA GA

of human (BC074029), mouse (BC116872), cat (AY117395), pig (AY526089), and horse (M14540), and IFN-β of human (M25460), mouse (BC119395), cat (AB021707), pig (NM_001003923), and horse (M14546). The PCR products were isolated by electrophoresis in an 1% agarose gel and purified using the Wizard SV Gel and PCR Clean-Up System (Promega). The purified PCR products were cloned into pCR-TOPO vector using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) and the sequence was determined using the Big Dye™ Terminator kit (ABI, CA, USA) and ABI PRISM™ 377 DNA Sequencer (ABI, CA, USA). We utilized the sequence data to design new primers for PCR amplification of the entire IFN-α and IFN-β cDNAs. Finally, we determined the ORF for the IFN-α and IFN-β cDNA sequences

```
  1 GGTTGAGAAACAGTGCTCTGAACCTATTTAGAAAGTGCATAAAGGAAAGCAAAAGCAGAA
 61 GTAGAAAGTGAGGGGAACATTCAGAAATTGAGAACTCACATGTTCCCTATATATGATACA
121 TGCACAGAGGCAGGTCTTCACAACCTAGAGCCCAAGGTTGGCAGTGTCATCCAGCGCAGC
181 CAGGCCGGCCTCATCTGCAAGATCTCCAATGGCCCTGCCCTGTTCCTTCCTGATGGCCGT
                              M  A  L  P  C  S  F  L  M  A  V   11

241 CGTGGTGCTCAGCTGCCACTCCATCTGCTCTCTGGGCTGTGACCTGCCTCTGACCCACAG
     V  V  L  S  C  H  S  I  C  S  L  G  C  D  L  P  L  T  H  S   31

301 CCTGGTCGACAGGAGGGCGTTGATCCTTCTGGGACAAATGAGGAGAATCTCTCCTTTCTC
     L  V  D  R  R  A  L  I  L  L  G  Q  M  R  R  I  S  P  F  S   51

361 CTGCCTCAAGGACAGAGAAGACTTTGGATTCCCCCAGGGGGTGCTTCATGGCAACCAGTT
     C  L  K  D  R  E  D  F  G  F  P  Q  G  V  L  H  G  N  Q  F   71

421 CCAGGAGGCTCAAGCCATCGCTGTTGCCCACGAGGTGACCCGGCAGACCTTCCTCCTCTT
     Q  E  A  Q  A  I  A  V  A  H  E  V  T  R  Q  T  F  L  L  F   91

481 CTGCACAGAGGCCTCATCCGCAGCTTGGGATGAGACCCTGCGAAGCAGATTCTGCACTGG
     C  T  E  A  S  S  A  A  W  D  E  T  L  R  S  R  F  C  T  G  111

541 ACTCTATCAGCAGCTGATCCACCTGGAAGCCTGTCAGACGCGGGAGGTGGGGGCGGAGGA
     L  Y  Q  Q  L  I  H  L  E  A  C  Q  T  R  E  V  G  A  E  E  131

601 GACTCCCCTGCTGGATGAGGACTCCACACTGGCTGTGAGGAGTTACTTCCAGAGACTCTT
     T  P  L  L  D  E  D  S  T  L  A  V  R  S  Y  F  Q  R  L  F  151

661 CCTCTATCTGCAGGAGAAGAAACACAGCCCTTGTGCCTGGGAGATTATCAGAGCAGAGAT
     L  Y  L  Q  E  K  K  H  S  P  C  A  W  E  I  I  R  A  E  I  171

721 CATGAGGTCCTACTCTTTATCAACACACTTGAAGGAAACCAAGGAGTAAGGATTGACACT
     M  R  S  Y  S  L  S  T  H  L  K  E  T  K  E  *              186

781 GGTTCAACATGGAAATGCTTCTC
```

Fig. 1. The nucleotide and deduced amino acid sequences of bat IFN-α (A) and -β (B). The numbers at left indicate the leftmost nucleotide position. The numbers at right indicate the rightmost amino acid position. Amino acid residues are shown by the one-letter abbreviation code based on the nucleotide sequence. Nucleotides in the 5′ and 3′ non-coding regions are shown preceding the ATG (start methionine codon) and following the TGA or TAA (stop codon, indicated by *), respectively.

```
   1  GTAGATAGTTGAGATTGCCAGACAATGCACTGGTGACTGACAGACAATGCACTGAGTAGA
  61  TAGTTGAGATTGCCAGAGGGGAGGGGGTTGGGGGAAATGGGTGAAAAAGGTGAAAGGGAT
 121  TAAGAAGTTCAAATTACCAGTTATAAAAATAGTCATAGGAATGTGAAGTACAGCATAGGG
 181  AATATAGTCAATAATGATGTAATAACTATGTATGGTGCCAGATGGGTACTAGATTTTTTG
 241  GGGTGATCACTTCATATGGTATATAAATATCTAACCACTATGTCGTACATCTAAAACTGA
 301  TATGATTTTCATTCCATTGTAATTGAAAAATATAAATGACAAAAGAAAACTGAAAAGGAG
 361  AACTGAAAATGGGAAATTCCTCTGAAATAGAAAGGGTTGATGACCGTATAAATAGCCCAG
 421  GCTCATGGAGAAAAGACATTCACACTGCCAACACTTGAAGCCTTCCCTTCAGTGCCTAGG
 481  TGATAGTAGGCACCACTGTTCCCGTTTTCACCATGACCAACAGGTGCATCCTCCAAATTG
                                  M  T  N  R  C  I  L  Q  I  A  10

 541  CTCTCCTGTTGTGTTTCTCCACCACAGCTCTTTCCATGAGCTACGACTTGCTTCGATTTC
        L  L  L  C  F  S  T  T  A  L  S  M  S  Y  D  L  L  R  F  Q  30

 601  AACAAAGAAGCAGCAATTTAGCCTGCCTGAAGCTCCTGTGGCGGTTAAATGGAACCCCTC
        Q  R  S  S  N  L  A  C  L  K  L  L  W  R  L  N  G  T  P  Q  50

 661  AATATTGCCTCAAGGACAGGATGGACTTCAAGATCCCTGCGGAGATTAAACAACCACAGC
        Y  C  L  K  D  R  M  D  F  K  I  P  A  E  I  K  Q  P  Q  Q  70

 721  AACTCCAGAAGGAGGACGCAGTATTGATCATCCATGAGATGCTCCAGCAGATCTTTGGTA
        L  Q  K  E  D  A  V  L  I  I  H  E  M  L  Q  Q  I  F  G  I   90

 781  TTCTCCAAAGAAATTTCTCTAGCACTGGCTGGAATGAGACCATCATTGAGACTCTCTTTG
        L  Q  R  N  F  S  S  T  G  W  N  E  T  I  I  E  T  L  F  V 110

 841  TGAAACTTGATAAGCAGATAGACCTTCTGGATACAGCCCTGGAGAAACTGGAGAAGGAAA
        K  L  D  K  Q  I  D  L  L  D  T  A  L  E  K  L  E  K  E  N 130

 901  ACTTCACCTGGGAAAGCATGACAGTGCTGCACCTAAAGAATTACTACTTTAGGATCATGA
        F  T  W  E  S  M  T  V  L  H  L  K  N  Y  Y  F  R  I  M  R 150

 961  GGTACCTGAAGATCAGGTTGTACAGCAGATGTGCCTGGACAGTAGTCCGAGCGGAAATTC
        Y  L  K  I  R  L  Y  S  R  C  A  W  T  V  V  R  A  E  I  L 170

1021  TCAGGAACTTTTCCTTCCTTATTGGACTTACAGAATACCTCCGAAACTGAAGATCTCTCC
        R  N  F  S  F  L  I  G  L  T  E  Y  L  R  N  *            185

1081  GCCTGTACTTCTGAGAAAGAACAGTGCTTG
```

Fig. 1. (*Continued*).

using IFN-α F3, R2 and IFN-β cdsF, cdsR (Table 1). The ORFs and the deduced amino acid sequences were analyzed using the genetic information processing software GENETYX-WIN Version 4.0.2 (Software Development, Tokyo, Japan).

2.3. Phylogenetic analysis of IFNs

Mammalian IFN nucleotide sequence information was obtained from GenBank. Species in the phylogenetic tree were limited because only several species

have enough numbers of subtypes of known INFs sequences. Sequences were aligned using Clustal W (Version 1.83; http://www.cf.ac.uk/biosi/research/biosoft/downloads/clustalw.html), checked by eye, and all positions with gaps or ambiguous alignments were excluded from the analysis. A phylogenetic tree was constructed using Phylip (Version 3.6.5; http://evolution.genetics.washington.edu/phylip.html) with the following full-length IFN ORFs referred to GenBank: human (IFN-α1 (NP_076918), IFN-α2 (NP_000596), IFN-α5 (NP_002160), IFN-α14 (NP_002163), and IFN-β (AAC41702)), horse (IFN-α1 (P05003), IFN-α2 (P05004), IFN-α3 (P05005), IFN-α4 (P05006), and IFN-β (P05012)), pig (IFN-α1 (NP_999558), IFN-α3 (ABI26095), IFN-α10 (ABB51634), IFN-α14 (ABB51627), and IFN-β (NP_001003923)), dog (IFN-α1 (P81255), IFN-α5 (BAD18111), IFN-α7 (NP_001006655), IFN-α8 (NP_00100713) and IFN-β (XP_538679)), cat (IFN-α1 (AAM78030), IFN-α7 (BAC75983), IFN-α10 (NP_001027000), IFN-α14 (NP_001027002), and IFN-β (BAA93629)), mouse (IFN-α1 (AAO63592), IFN-α5 (AAI20911), IFN-α7 (NP_032360), IFN-α14 (NP_996858)), chicken (IFN-α (BAA83090) and IFN-β (NP_001020007)).

2.4. Preparation of bat primary kidney cells and bat cell line

Fresh kidney of *Rousettus leschenaulti* under anesthesia with ketamine (5 mg/ml/kg) and medetomidine (0.2 mg/ml/kg) were removed, sliced and treated with 0.25% Trypsin–EDTA in phosphate-buffered saline (PBS). Whole blood of bat was collected by heart puncture under anesthesia. Collected bat primary kidney cells were seeded on 10-cm^2 plate in Dulbecco's modified eagle's medium (DMEM) (Invitrogen) with 5% heat-inactivated fetal calf serum (FCS). Bat lung epithelial cell line, Tb-1 Lu, was maintained in incubation of 5% CO_2 at 37 °C in DMEM containing 10% FCS.

2.5. Expression of type I IFNs mRNA using poly(I:C) treatment

BPKCs and Tb-1 Lu cells were treated with 5% FCS-DMEM including 10 μg/ml poly(I:C) (Sigma, St. Louis, MO) and 150 μg/ml diethylaminoethyl dextran (DEAE-Dextran) (Sigma) in 5% CO_2 at 37 °C for 3 h. Cells were then washed twice with PBS and total RNA was isolated using ISOGEN solution (NIPPON GENE, Toyama, Japan).

2.6. Preparation of bat type I IFNs-containing medium

BPKCs were treated with 5% FCS-DMEM including 10 μg/ml poly(I:C) and 150 μg/ml DEAE-Dextran in 5% CO_2 at 37 °C for 3 h. After treatment, the cells were washed twice and cultured in fresh 5% FCS-DMEM for 24 h. The whole supernatant, bat type I IFNs-containing medium, was collected and stored at 4 °C until usage.

2.7. Expressions of bat IFNs mRNA under bat type I IFNs treatment

BPKCs and Tb-1 Lu cells were prepared at 5×10^5 ml^{-1} in 6-well culture plate containing 2 ml per well for 2 days, and then treated with 200 μl of bat type I IFNs-containing medium for 1 h. After that, cells were washed three times and then incubated in 5% CO_2 at 37 °C in DMEM containing 5% FCS (primary kidney cells) or 10% FCS (Tb-1 Lu cells) for additional 0, 4, and 8 h. After incubation, total RNA was isolated from these cells with ISOGEN solution (NIPPON GENE).

2.8. Reverse-transcription PCR analysis of type I IFNs mRNA expression

Total RNAs were treated with DNase I (Takara Bio) according to the manufacturer's instructions. RNA samples were then reverse-transcribed using the $Oligo(dT)_{12-18}$ primer and SuperScript™ II (Invitrogen) for synthetic first-strand cDNA. cDNAs were used as a template for semi-quantitative PCR with TaKaRa Ex Taq using GAPDH F and R, IFN-α F3 and R2 and IFN-β cdsF and cdsR (Table 1) as primers. The PCR products were analyzed by 1% agarose gel electrophoresis and stained with ethidium bromide.

In this study, the experiment was performed in accordance with the Animal Experimentation Guideline, the University of Tokyo, and was approved by the Institutional Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, the University of Tokyo.

3. Results and discussions

3.1. Cloning of full-length bat type I IFN ORFs and phylogenetic analysis of IFNs

The nucleic acid and amino acid sequences of one of the IFN-α genes and the IFN-β gene from *R. aegyptiacus* were determined. The full-length bat IFN-α ORF was 562 bp and encoded 187-aa polypep-

tides. Bat IFN-β ORF was 558 bp and encoded 186-aa polypeptides (Fig. 1(A and B)). Direct comparison of bat IFN-α genes simultaneously against various animal species was complicated by the fact that there are various IFN-α subtypes and difficulties in the identification of subtype of the bat IFN-α. Therefore, sequence comparisons were performed using IFN-β between bat and human, pig, cat, horse, and mouse. The identity of bat IFN-β with human, pig, cat, horse, and mouse IFN-β were 77.5, 82.0, 78.3, 77.5, and 66.5% at the nucleotide level and 64.2, 72.0, 61.8, 61.3, and 49.5% at the amino acid level, respectively. Phylogenetic analysis using the amino acid sequences from several representative eutherian type I IFNs and chicken type I IFN found that both bat IFN-α and bat IFN-β are homologous to the mammalian IFN group. Further analysis showed that both bat IFN-α and IFN-β were most closely related to those of pig, and followed by horse (Fig. 2). Although phylogenetic relationship between bat and other animals remains inconclusive, molecular phylogenetics using mitochondrial DNA or retro-transposon insertions indicated that Chiroptera is included in Fereuungrate or Pegasoferae (Perissodactyla, Carnivora, Pholidota, and Chiroptera) (Nikaido et al., 2000; Nishihara et al., 2006). Comparison of the amino acid sequences of the cell surface molecule CD4 showed that bat is more closely related to cat and dog (Omatsu et al., 2006). Our findings and these molecular phylogenetic analyses suggested that bat might have anti-viral mechanism similar to these animals. Some investigators indicated that Nipah virus spread from Megachiroptera to pig and then from pig to human (Tan and Wong, 2003), and pig might be more susceptible to the virus than other animals. In contrast, relatively low

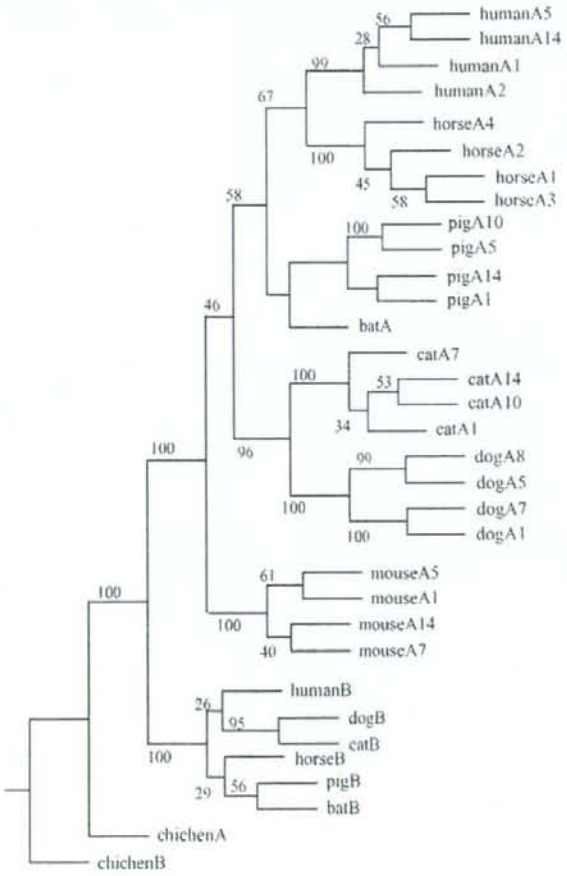

Fig. 2. Maximum likelihood phylogenetic tree constructed by the Phylip 3.65 program using amino acid sequences from human, horse, pig, cat, dog, mouse, chicken, and bat type I IFNs. The numbers at the nodes indicate bootstrap values. 'A' and 'B' reflect IFN-α and -β, respectively.

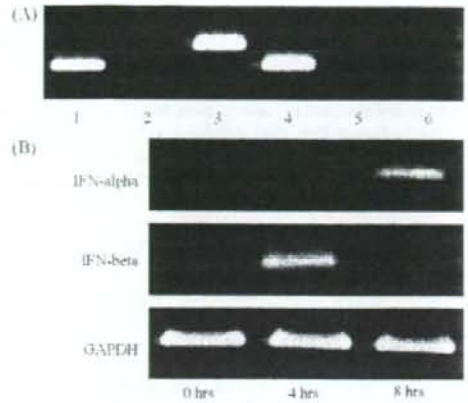

Fig. 3. Expression of type I IFNs in BPKCs and Tb-1 Lu. (A) Expression of type I IFN mRNAs in response to poly(I:C) treatment. IFN-α mRNA (lanes 2 and 5), IFN-β mRNA (lanes 3 and 6), and GAPDH control mRNA (lanes 1 and 4) from BPKCs (lanes 1–3) and Tb-1 Lu cells (lanes 4–6) were analyzed using semi-quantitative reverse-transcription PCR of total RNA followed by 1% agarose gel electrophoresis and ethidium bromide staining. (B) Temporal change of type I IFN mRNAs in BPKCs after treatment with bat IFNs-containing medium. IFN-α mRNA (upper panel), IFN-β mRNA (middle panel) and GAPDH mRNA (control; lower panel) from BPKCs were analyzed following treatment with bat IFNs-containing medium for the indicated period of time.

homology of immune factors between bat and human suggested the presence of different anti-viral activity against some viral infections. These factors might be one of the key factors to control zoonoses from bat.

3.2. Expression of bat type I IFN

To investigate whether BPKCs and Tb-1 Lu cells have the capacity of IFN production, we first examined the up-regulation of type I IFNs in response to poly(I:C) treatment. In BPKCs, there is an increase in the expression of IFN-β mRNA, but not IFN-α mRNA, at 3 h after poly(I:C) treatment. However, in Tb-1 Lu cells, poly(I:C) treatment induced IFN-α mRNA production but production of INF-β mRNA was not observed (Fig. 3(A)). To examine whether BPKCs or Tb-1 Lu expresses type I IFN mRNA in response to the bat IFN-containing medium (exogenous IFN), the expression of type I IFNs mRNA was examined at 0, 4, and 8 h after the exogenous IFN-treatment. In the case of BPKCs, IFN-α mRNA was detected at each time point with a gradual increase, and IFN-β mRNA which was not initially detected, peaked at 4 h in response to exogenous IFN (Fig. 3(B)). In Tb-1 Lu, however, type I IFNs mRNA expression was not detected at all (data not shown). Although BPKCs could induce type I IFNs mRNA in response to poly(I:C) via TLR3, RIG-I, and mda-5, type I IFNs-inducing signal was not sufficient for the stimulation of IFN-α mRNA synthesis. In contrast, when type I IFNs were supplied to BPKCs, IFN-β mRNA was induced more rapidly than IFN-α. This indicated that IFN-β is involved in immediate response to invasion of viruses or microbes and IFN-α, which is responsible for anti-viral activity via stimulation of PKR, OAS, and Mx synthesis, is induced by IFN-β and has more prolonged response in BPKCs (Fig. 3(B)). When Tb-1 Lu were treated with either poly(I:C) or bat IFNs-containing medium, these cells did not express any IFN-α or IFN-β mRNA. This suggests that in Tb-1 Lu the mechanism of dsRNA recognition or the signaling pathway reacted to exogenous IFNs is not utilized for up-regulation of type I IFNs mRNA. Thus, the IFN signal responding to both poly(I:C) and exogenous type I IFNs was different between BPKCs and Tb-1 Lu. Bat is diversified into about a thousand species in the world. These results further indicated that extra considerations should be taken in the interpretation of experimental data of anti-viral dynamics among various bat cell types and species.

The bat immune system is of particular interest because of its ability to act as a reservoir for a variety of pathogens that pose serious health threats to humans. However, these studies are complicated because few studies on anti-viral mechanism of bat are available. Thus, the nucleotide sequences of type I IFNs of Rousette bat were characterized for the first time. To investigate whether and how bats harbor clinically important pathogens, some basic information from inoculation studies performed *in vivo* and *in vitro* is very important. To determine how wild animals remain asymptomatic to pathogens, it will be necessary to understand their viral control mechanisms, such as IFN signaling. Using bats as representative pathogenic carriers, this study provides some basic and important immunological information about bat. It is necessary for understanding zoonoses from bat, especially for Megachiroptera.

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Current Status and Measures of Zoonosis Control in Japan

Yasuhiro YOSHIKAWA

Graduate School of Agricultural and Life Sciences, The University of Tokyo
1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
e-mail: ayyoshi@mail.ecc.u-tokyo.ac.jp

Abstract

The main cause of human infectious diseases and public health threats these days has been thought to be "emerging and re-emerging diseases" such as BSE, highly pathogenic avian influenza (HPAI), SARS, etc. These infectious diseases were derived from pathogens from animals (zoonosis) and their products (food-borne infections). Recent zoonoses worldwide are reviewed here by origin, environment and relationship to human activities both in developing and developed countries. Emerging zoonosis is a kind of warning to humanity and we need to build ubiquitous, scientific zoonosis control methods acceptable to most countries of the world. The WHO and OIE serve as internationally responsible organs for disease control in humans and domestic animals, respectively. I explain a path to global zoonosis control, recent experiences in risk assessment and new measures for zoonosis control in Japan, which have been conducted concomitant with renovation of the "Infectious Diseases Control Law" which was established about a hundred years ago. The renovated law calls for cooperation between central and local government, between the MAFF (Ministry of Agriculture, Fishery and Forestry) and MHLW (Ministry of Health Labor and Welfare), and between medical doctors and veterinarians.

Key words: conservation medicine, domestic animal, OIE, risk assessment, WHO, zoonosis

1. Introduction

Recently, the cause of many human infectious diseases posing a public health threat is considered to be derived from so-called "emerging and re-emerging diseases." These diseases are caused mainly by pathogens originating from animals (zoonoses) and their products (food born infections). Particularly in tropical countries, there are many factors causing an emergence of infectious diseases. These include the pathogenic agents themselves, their reservoirs (natural hosts) and vectors (intermediate hosts), which may be resident in nature. The acceleration of expanding international trade enhanced by the World Trade Organization (WTO), especially in food products and exotic pet animals may produce newly emerging diseases. Moreover, the aging populations of the developed countries, increasing the number of people who are potentially more susceptible to opportunistic infections, is also connected with an increase in outbreaks of emerging and re-emerging diseases. A wide variety of animal species, both domesticated and wild, has acted as reservoirs for these pathogens, which include viruses, bacteria and parasites.

In nature, human beings are heterotrophic organisms that depend on animals and plants as sources of nourishment. Most of our needs for protein and fat are supplemented by consuming the milk, meat, internal organs and other parts of domestic animals. Thus, we have had a long relationship with domestic animals, some of which were already living among us when our ancestors started culturing the land 10,000 years ago. A look at that history shows that almost all current infectious diseases suffered by humans have had animal origins. In other words, diseases such as smallpox, measles and influenza that were once thought to be unique to human, all either originated in other animals or share common ancestors with viruses infecting other animals. For example, the smallpox virus is a relative of the cowpox virus, the measles virus originated from rinderpest or canine distemper viruses, and influenza virus is derived from avian influenza viruses. There are also many infectious diseases even today that can be transmitted between people and domestic animals. We humans do not inhabit a special world separate from that of other animals.

2. Diseases from Animals to Humans

Zoonotic infections are diseases caused by a pathogen that infects both animals and humans (but natural hosts infected by the pathogen often do not suffer adverse effects or sickness). They consist mostly of diseases passed on to humans from animals. Some zoonoses, however, originally passed on to animals from

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