キンカジューを安全に飼育するために（案）

～キンカジュー回虫のリスクとその回避方法に関するガイドライン～

概要

（箇条書きで、全体概要をシンプルにまとめる）

本編

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（本ガイドライン作成の目的、対象者等について）

I. 背景

1. キンカジューの概要

1.1　キンカジューとは

（キンカジューの分類、生態、ペットとしての飼育ブーム、等の基礎情報）

1.2　注意すべき人獣共通感染症

（CDC の警告、アライグマ回虫の問題、ヒト症例の紹介[脳にくるのでリスクが高い]）

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（輸入状況、飼育状況[動物園など]、日本でのペット飼育例）

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（動物園や獣医師による実例、実績等）

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（実験結果について解説）

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（糞便検査方法や確定診断のための連絡先等）

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（寄生虫検査、検査結果後の対応等のフロー図をわかりやすくまとめる）

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2. 獣医師
3. 動物園飼育者
4. ペット所有者

おわりに

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（関連、参考となるガイドライン等）

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おわりに

海外委託

フィリピン大学／ジョセフ・マサンガイ

Feature

On Surveying Bats And Caves

By JOSEPH S. MASANGKAY, DVM, PHD,
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ROBERTO P PUENTESPINA, JR., DVM

People who climb mountains are called mountaineers, those who dive are called divers, and those who walk long distances are called hikers or trekkers. How about those who explore caves? They are commonly called cavers, but the more technical term is "spelunkers". In our past scientific expedition which covered only one bat cave in the Island Garden City of Samal (IGaCOS) in Davao and reported in the past issues of Animal Scene, we described the unique behavior pattern of the bats occupying this cave. One of the main purposes of our scientific expedition is to take biological specimen samples (blood and organ tissues) from bats and examine these samples for potential zoonotic pathogens. Unfortunately, in spite of having a permit from the local Department of Environment and Natural Resources (DENR) the foundations managing the cave located in a private land did not allow us to take specimen samples from the bat colony of Rousettus amplexicaudatus. We decided not to pursue such kind of investigation in this particular cave but concentrate our effort, energy, resources, and time for the other bat caves on Samal island and neighboring smaller islands like Talikud island.

For safety, the passengers of the boat must wear the life jacket by himself as a try-on try-off self demonstration.

Transportation in remote rural areas with very narrow roads uses motorcycles. Passengers, one or two ride tandem with the driver and called "habal-habal." In places with wider road, a plank of wood is attached across to the back seat and here the passengers ride up to more than 6 persons. Because of the shape it was given the moniker "skylab." If you see one, you will be amazed by the ability of the driver to balance the motorcycle while moving.

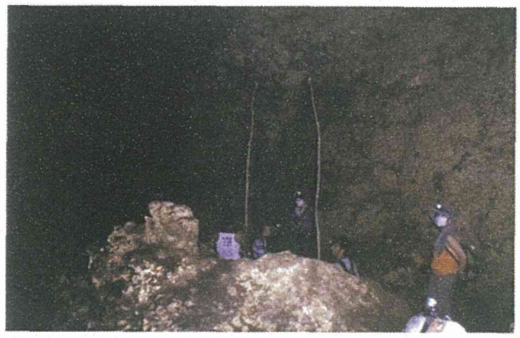
To make legal and legitimate our scientific expedition we secured a GP (Gratuitous Permit) from the local office of the DENR which allowed us to do our specimen collection. The permit was issued under the name of Ms. Ma. Regina M. Quibod (Wildlife Gratitous Permit-WGP No. XI-2011-06) effective until October of this year 2012. Ms. Quibod's MS thesis is on "Cave survey, assessment and bat profiling". Although Dr. Joseph S. Masangkay is a regular faculty member of the UPLB College of Veterinary Medicine he is also an affiliate faculty member of the UPLB College of Arts and Sciences and serves as thesis panel members for graduate students doing their studies on wildlife including Ms. Quibod. >>

This picture shows the bat team disembarking from the boat. Take note that boats with outrigger have a shallow draft enabling them to come closer to shore without need for piers.

Ms. Nina Quibod, a graduate MS student in Wildlife at UPLB, is seen here giving a pre-entry briefing to the bat team before entering the cave. Silence must be observed while inside the cave to avoid disturbing the bats.

Dr. Yoshikawa is shown riding in tandem on a motorcycle called "habal-habal". Persons with *no experience riding bicycles or motorcycles will* be very difficult to have as passengers because of uncoordinated movement with the driver. The naive passenger will try to counter or anticipate the turns, *curves and dips making the ride difficult* for the driver to balance the motorcycle.

This is the entrance to the Baga cave on Talikud island. It is given the name Baga, which is the Tagalog word for "lung" because as one enters the cave and goes deeper and deeper the air becomes thin and the oxygen concentration drops, *making breathing difficult. The name is very appropriate since the cave will really* "test" whether your lung can function efficiently.



The ceiling of the cave is high and our poles were much shorter making it difficult for us to catch the bats.

The ceiling of the cave is high and our poles were much shorter, making it difficult for us to catch the bats.

The Samal Island group includes a much smaller island called Talikud island. Talikud or Talikod is Tagalog for "at the back of" because it is situated at the south end of the main island of Samal. See the map at the end of this article. Aside from bat caves, the island is also famous for beach resorts with white sand and diving areas that can compete with the most famous island resorts for tourism. We rented a motorized boat with outriggers from Davao Sta. Ana pier up to Islareta resort which served as our temporary base camp where we left our things and belongings and also served as our resting and eating place. Transportation within the island of Talikud is limited to the motorcycle. It is a system of tandem riding with one or two persons riding together with the driver. This is a very unique way of transportation in remote rural areas and called "habal-habal". In areas where the road is a little bit wider, the driver attaches a plank of wood across the back seat and here people ride on both sides of the plank that can accommodate up to more than six persons depending on the body size of the passengers. Because of its unique shape, it is called "Skylab." Riding a la-tandem is not for the faint-hearted since it requires balance and agility. The driver will have a hard time if the tandem rider does not have any experience riding bicycle because the tendency for naive riders is to counter or anticipate turns and dips along the road jeopardizing the stability of the motorcycle.

Before the group entered the "baga" cave, Nina Quibod gave some orientation pointers to observe while inside the cave, such as minimizing noise so as not to disturb the bats. The name "baga" which is the Tagalog term for "lung" is very appropriate because as you go deeper and deeper into the cave the air becomes thinner and the oxygen concentration decreases making it difficult to breath, hence, the cave tests whether you have lungs that can withstand the thin air with low oxygen concentration. The ceiling was also high and the poles we used to attach the net were short, making it difficult to catch the >>

Dr. Bo Puentespina is shown here with a pile of freshly cut trees ready for charcoal-making. This is a familiar scene in most forests in the country and although illegal is not strictly prohibited. Such practice is not good to the ecological balance and can even lead to soil erosion and forest degradation.

Dr. Maeda is shown here blowing the candles on a specially-baked chocolate cake. He got a real surprise of his life when he did not expect us to remember his birthday.

bats. After a little more than one hour, we were able to catch only a few bats. We considered the catch, although few in numbers a lucky catch because it included Rousettus amplexicaudatus bats which are actually our target species. In our past scientific expeditions in different places in Luzon and Visayas we only caught two bats belonging to the Rousettus amplexicaudatus. Both bats tested negative for Reston Ebola antibodies. In other deadly Ebola infection in Africa that includes virus from Sudan, Ivory Coast, Zaire and other places the virus had been proven to be carried by Rousettus aegyptiacus species of bats. Reston Ebola virus (REBOV) on the other hand had shown to be non-pathogenic for pigs and humans. The virus becomes deadly only in long-tailed macaque Macaca fascicularis. This is the reason we are interested in catching bats belonging to the Rousettus amplexicaudatus species. A cave to be considered "live" must contain different species of animals that include not only bats but also reptiles like geckos or even snakes, particularly pythons. These higher forms of vertebrates feed on some other vertebrates. The resident python is very lucky because it does not need to hunt. It is as if every day for the python is an "eat all you can" smorgasbord meal. However pythons have slow metabolic rate and do not have to eat everyday. One bat consumed by the python is enough for several days before it gets hungry again.

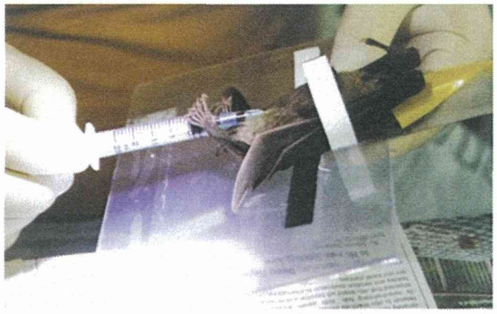
Insects and arachnids form the lower echelon of cave hierarchy. The guano from fecal droppings of bats serve as good medium to sustain invertebrates like cockroaches, coprophagus beetles, and mites. If a baby bat falls on the floor of the cave, it is doomed because it will be preyed upon by the different invertebrates.

After returning to our base camp at the Islareta beach resort, preliminary examination was done on the captured bats, especially those that were weak. Euthanasia using anaesthesia injected intraperitoneally was done and blood extraction was also immediately done by intracardiac route. After a sumptuous lunch, some rested while others opted to take a quick dip at the inviting crystal-clear sea. Taking a bath in sea water after entering a bat cave is also a good practice to disinfect the body of possible bacterial and viral pathogens from contamination with bat urine and feces. The sodium chloride (table salt) in sea water is a very effective disinfectant that can kill most common commensal bacteria. On our way back to the mainland, most of us were exhausted and we had a nice nap stretched on the long bench of the boat. Upon reaching the Malagos Garden Resort where we stayed during our expedition, we identified the flyway of the bats in between open spaces of trees and also the flyway in the creek. Before dark we

The Malagos Garden Resort has many fruit trees very ideal for setting up mist nets. The place to set up the net is critical because one has to identify the flyway of the bats for the trapping to succeed.

were able to finish setting up the mist net. One of the professors, Dr. Ken Maeda of Yamaguchi University did not know that we knew it was his birthday. While we were at Islareta resort Dr. Bo Puentespina called the chef of the resort to bake a chocolate cake for Dr. Maeda. Just before dinner, Dr. Maeda got the surprise of his life when we sang happy birthday to him and requested him to blow the candles. He said that it was the first time for him to have such a birthday surprise. After dinner, we checked the mist net. The identified flyways were excellent since we were able to catch a lot of bat species including the rare nectar-feeding bat. The function room in the resort was graciously

The trapping in Malagos Garden Resort was a huge success. Here Ed Eres, a staff of the Museum of Natural History, UPLB, is removing the captured bat from the net assisted by Ms. Nina Quibod.

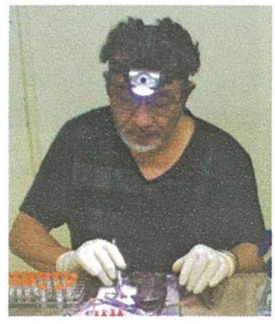


This anesthetized bat is held in a restraining contraption fabricated by Dr. Une. Such kind of restraint is necessary to avoid injury to both the bat and the person examining it. Here, blood is being collected intracardially.

offered by the resort owner to serve as our laboratory dissecting room. Just like in past expeditions there was a division of labor not different from an assembly plant of some Japanese cars. The Museum of Natural History staff which includes Nina Quibod, Edison Cosico, Ed Eres and Ariel Larona were the first in the line who identified the dead bats using the standard table for taxonomic classification of bats based on morphometric measurements of the anatomical structures such as leg and arm length. External morphological features such as presence of epaulet and shape and length of the ears were also considered for precise taxonomic classification. Live bats were first restrained inside plastic bags for body weight measurement using sensitive digital portable weighing scale. From the body weight the anesthetic dose was computed. Once the bat becomes immobilized due to anesthesia given by intraperitoneal route, it is restrained in a contraption fabricated by Dr. Yumi Une. Blood was extracted by intracardial route which effectively euthanized the animal. The collected blood was centrifuged using portable microcentrifuge to separate the serum. External parasites were collected for future identification. Dissection commences until all the needed internal organs were collected including liver, spleen, kidney, and brain. From the collected tissue, organs different biomedical examination will be done such as bacteriological isolation, virus examination and biomolecular extraction of RNA and DNA for further amino acid

This is the nectar-feeding bat, a rare specimen caught in Malagos Garden Resort. Take note of the protruding tongue which can be stretched thinly enabling the bat to suck the nectar from flowers and fruits.

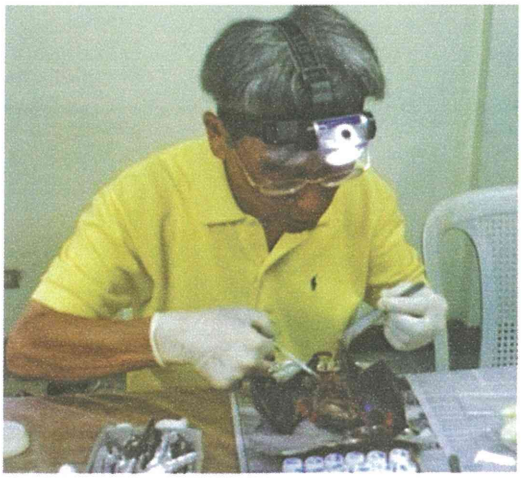
A fruit bat shown here can easily be caught in areas with fruit-bearing trees, just like in the Malagos Garden Resort. Take note of the big eyes which they use for navigation. Fruit bats have less ability to echolocate and can easily be caught using the mist nets. On the other hand, the insectivorous bats with their cunning ability to echolocate are difficult to catch because they can easily detect and avoid the mist nets.



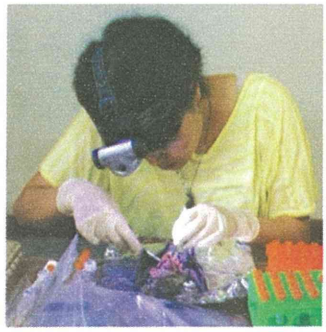
Dr. Yasuhiro Yoshikawa, the overall leader of our bat team and retired emeritus professor of The University of Tokyo is shown here dissecting the bat. At present, he is now connected with Chiba Institute of Science as Vice president.

The Malagos Garden Resort which served as our headquarters allowed us to use its function room as our laboratory. Here the staff of MNH-UPLB, Nina, Ed and Edison Cosico is the first to examine the bats for identification and taxonomic classification.

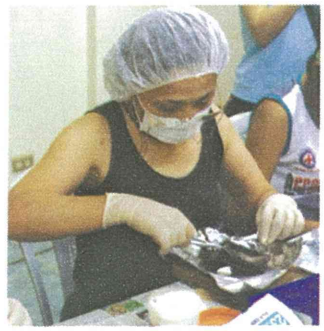
Dr. Ken Maeda of Yamaguchi University is labelling the specimen container for accurate identification. Dr. Maeda's line of research is immunology and has developed a non-invasive method of examination just by collecting feces and urine from bats for immunological assay.



Dr. Hiroomi Akashi, a retired professor of the University of Tokyo, is collecting tissue samples from the bat for his research in microbiology.



Dr. Yukiko Sassa of Tokyo University of Agriculture and Technology is shown here dissecting the bat. Dr. Sassa was a former special exchange student who studied for one year at the UPLB College of Veterinary Medicine.



Dr. Yumi Une of Azabu University is injecting anaesthetic to the bat intraperitoneally in compliance with the rules and regulation of the Animal Welfare Act to enable the scientists to examine the bats without causing undue pain.

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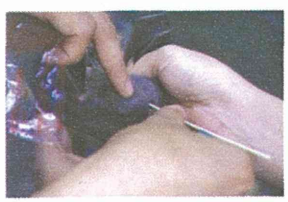
sequencing. Collected samples were put in liquid nitrogen gas to preserve the specimen which will be further examined in the laboratory. The bat carcasses were fixed in 10 % formalin solution to be stored at the Museum of Natural History-UPLB for archiving and future reference.

The last site for our specimen collection was the Manan-ao cave in the Island Garden City of Samal (IGaCoS). The features of this cave are very different from Baga cave since the approach was very steep and the entrance was fenced off by a cyclone fence to ward off illegal bats and guano collectors. Only the younger students were allowed to enter the cave using rappelling rope to be able to go down and up the vertical wall of the cave. It was a complete vertical drop measuring 40 meters down to the floor of the cave. The cave was small and we were able to capture many bats mostly Rousettus amplexicaudatus species. The final headcount of captured bats from different sites totalled 71 bats belonging to four species as shown in the table below.

Number of bats captured from three sites
(Baga cave, Malagos Garden Resort and Manan-ao cave)

Species of bats	Number of captured bats
Rousettus amplexicaudatus	40
Ptenochirus jagori	7
Cynopterus brachyotis	19
Macroglossus minimus	4
Rhinolopus rufus	1
TOTAL	71



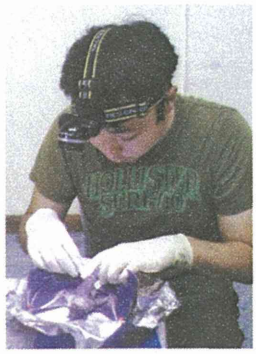
Rectal swab is another method of specimen collection without having to sacrifice the animal. After specimen collection, the bat can then be released unharmed.

Dr. Akashi had just come out of the cave carrying the bird bags that contained the captured bats.

Map of Samal Island shown with the smaller island named Talikud island where the first cave (Baga cave) we surveyed is located. This island is a resort area famous for its white beach and excellent dive haven.

The dissected bat carcass is placed inside resealable plastic bag containing 10% formalin solution. The collected carcasses are properly labelled for archiving at the UPLB-MNH.

Dr. Une is giving sugar water solution (SWS) orally to the bat before it will be released. Fruit bats have rapid rate of metabolism and if they stay overnight entangled in the mist net they can easily suffer from hypoglycaemia. Oral rehydration with SWS helps a lot to restore energy before releasing the bats.



Hiroshi Shimoda, a graduate student of Dr. Maeda, is collecting specimen samples from the bat. Mr. Shimoda studied in the USA for two years and is very fluent in English making communication with the other Filipino team members easy.

Dr. Akashi and Dr. Yoshikawa are negotiating the very steep footpath leading to the entrance of Manan-ao cave in Samal Island, Davao.

This is the final group picture of the bat team in front of Malagos Garden Resort after a successful scientific expedition of cave survey, assessment and bat profiling in IGaCoS in Davao.

The entrance to Manan-ao cave is fenced off by cyclone wire to discourage bat hunters and guano collectors. The approach is a straight vertical drop approximately 40 meters up to the floor of the cave. A rappelling rope is necessary to enable easy downward and upward approach.

The professors showing the bird bags containing lots of captured bats from Manan-ao cave.

Everything we did was according to the Wildlife Act rules and regulation and we secured a transport permit to enable us to transport the collected specimens from Davao to UPLB in Laguna. All collected samples were shown to the local DENR office for inspection and for issuance of a transport permit. Liquid nitrogen gas is not allowed inside the airplane but we have to keep the specimen under frozen condition. To maintain the cold chain needed for good preservation of the specimen, dry ice was used to replace the liquid nitrogen gas. Specimen preserved on dry ice is allowed as check-on baggage in the plane. ■

Ⅳ.　業績資料集

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Detection of bat coronaviruses from *Miniopterus fuliginosus* in Japan

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Abstract Bats have great potential as reservoirs for emerging viruses such as severe acute respiratory syndrome-coronavirus. In this study, bat coronaviruses (BtCoVs) were detected by RT-PCR from intestinal and fecal specimens of *Miniopterus fuliginosus* breeding colonies in Wakayama Prefecture caves, where we previously identified bat betaherpesvirus 2. Two primer sets were used for the detection of BtCoV: one was for the RNA-dependent RNA polymerase (RdRp) region and the other was for the spike (S) protein region. Eleven and 73% of intestinal and fecal specimens, respectively, were positive for RdRp region, and 2 and 40% of those were positive for S protein region. Sequencing and phylogenetic analysis showed that the detected BtCoV belonged to the group 1 (alpha) coronaviruses. These data suggest that BtCoV is endemic in *M. fuliginosus* in Japan.

Keywords Bats · Coronavirus · *Miniopterus fuliginosus* · Virus detection

Introduction

An outbreak of severe acute respiratory syndrome (SARS) that was caused by a newly identified SARS coronavirus (SARS-CoV) occurred in China, Singapore, Vietnam, and other countries from 2002 to 2003; 8,098 patients were reported, 774 of whom died [1]. At first, SARS-CoV was considered to be derived from civets and raccoon dogs [2]. However, recent studies have suggested that SARS-CoV is a recombinant of bat-derived coronaviruses [3, 4]. Bat coronaviruses (BtCoVs) have now been identified in China [3, 4] and other countries such as USA [5], Germany [6], Kenya [7], and the Philippines [8]; however, there has been

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no report of BtCoV detection in Japan. Bats have also great potential as reservoirs for emerging viruses such as Ebola and Nipah [9, 10]. We have recently identified novel viruses from bats, such as bat betaherpesvirus 2 [11] and bat adenovirus 1 [12], in Japan. To confirm the role of bats as host species for viruses, we attempted to detect BtCoV from *Miniopterus fuliginosus* using intestinal and fecal specimens from specific caves located in the Wakayama prefecture, Japan.

Materials and methods

Bat samples

All bats and fecal specimens were collected in May 2009 and July 2010 from a headrace tunnel and a cave in the Wakayama Prefecture, Japan, with permission from government officials. Those are well-known habitats of *M. fuliginosus*; the headrace tunnel is a roosting place of male and non-breeding female bats and the cave is a breeding place, where we collected bats and identified a novel virus in a previous study [11, 13]. The following three methods were used to collect intestinal specimens: (1) Captured bats were euthanized using an overdose of anesthetic and small intestines were collected without washing their contents, (2) Forty-five bats were captured with a net and placed in a bag, after which fresh fecal samples were collected, placed in a solution of RNAlater (Ambion, Foster City, CA, USA), and separated into four pools. After fecal samples were collected, the bats were released safely, (3) Three traps were placed under a mass of bats that were hanging from the ceiling of each cave. The next day, fecal samples were collected, placed in RNAlater, and separated into 11 pools.

Extraction of RNA and RT-PCR

Total RNA was first extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and was re-extracted using the QIAampViral RNA Mini Kit (Qiagen, Hilden, Germany). The cDNA was synthesized using M-MLV Reverse Transcriptase (Invitrogen, San Diego, CA, USA) and random hexamers, and two-step reverse transcription PCR was performed using PrimeSTAR Max (Takara Bio, Shiga, Japan) with the default conditions of each master mix, except the annealing temperature, which was 48°C. Two primer sets were used for the detection of BtCoV; primer set for the first PCR: IN-6 primer, 5′-GGTTGGGAC TATCCTAAGTGTGA-3′ and IN-7 primer, 5′-CCAT CATCAGATAGAATCATCAT-3′ [14]; primer set for the second PCR: IN-6 primer and hemi-nested reverse primer, 5′-ATCAGATAGAATCATCATAGAGA-3′ [15], were used for detection of the sequence in RNA-dependent RNA polymerase (RdRp) region. These primer sets are known to be able to amplify genes of the coronavirus family including BtCoV [5, 8, 14, 16–18]. To amplify the spike (S) region sequence, consensus primers were constructed using the online consensus primer design software (CoCoMo; http://www.geneknot.jp/cocomo/) with seven sequences deposited in GenBank. The constructed primer sets for S protein region were as follows: No. 6, 5′-NSHRYKTATGTHTGYAAYGGHAA-3′ and No. 2 5′-DGAYTGBGAYTTDACACAYTCRTT-3′ were used for the first PCR; No. 1, 5′-HTGTGYBCAGYAYTAYAAY GGYAT-3′ and No. 2 were used for the second PCR. After amplification, amplicons were detected by 1.5% agarose gel electrophoresis.

Phylogenetic analysis

The phylogenetic analysis was performed as follows: obtained sequence data were carefully checked for peaks and 327 bp (RdRp) and 547 bp (S) of each amplicon, except primer sequences were selected to construct phylogenetic trees of nucleotide sequences. Five different sequences were obtained for the RdRp region (accession numbers AB619638–AB619642) and two sequences were obtained for S region (accession numbers AB644273 and AB644274). The same region of other available coronaviral sequences (BtCoV HKU7-1, DQ249226; BtCoV HKU8-1, DQ249228; BtCoV 1A, NC_010437; BtCoV 1B, NC_010436; BtCoV Fujian/773/2005, EF434379; HCoV 229E ATCC, NC_002645; HCoVNL63, NC_005831; PEDV CV777, NC_003436; AIBV Beaudette GK, AJ311317; MHV JHM, NC_006852; SARSr-Rh-BatCoV HKU3-7, GQ153542; SARS Frankfurt1, AY291315; civet SARSr-CoV PC4-139, AY613949; and EU420139, BtCoV HKU8) were used to create the phylogenetic trees. The bootstrap test for the phylogenetic tree was constructed using MEGA4 software [19] with the neighbor-joining method with a pairwise deletion option.

Results

As shown in Table 1, PCR products at the expected sizes were obtained from 5/45 (11%) of intestinal and 11/15 (73%) of fecal specimens with the RdRp primer set, and 1/45 of intestinal and 6/15 (40%) of fecal specimens were positive with the S protein primer set. PCR products were gel purified and sequence analysis was performed with specific-sequence primer. The sequences of PCR products showed some similarities to the deposited BtCoV sequences; these sequences were analyzed by a BLASTn search (http://blast.ddbj.nig.ac.jp/top-e.html) and matched to nucleotide sequences of BtCoVs. These suggest that the

Table 1 Detection of BtCoV from bat specimens

Region	Specimens	RT-PCR	Sequence type	Number	Total
RdRp	Intestine	5/45 (11%)	BtCoV M.ful./Japan/01/2009	4	5/45 (11%)
			BtCoV M.ful./Japan/02/2009	1	
	Feces from				
	Captured bat	2/4 (50%)	BtCoV M.ful./Japan/01/2010	1	11/15 (73%)
			BtCoV M.ful./Japan/03/2010	1	
	Trap	9/11 (82%)	BtCoV M.ful./Japan/01/2010	7	
			BtCoV M.ful./Japan/02/2010	2	
S	Intestine	1/45 (2%)	BtCoV M.ful./Japan/03/2009	1	1/45 (2%)
	Feces from				
	Captured bat	1/4 (25%)	BtCoV M.ful./Japan/04/2010	1	6/15 (40%)
	Trap	5/11 (45%)	BtCoV M.ful./Japan/04/2010	4	

detected sequences were derived from newly identified BtCoV sequences. Because the PCR product was detected not only in the feces pool but also in the small intestines, these results strongly suggest that *M. fuliginosus* in Japan is definitely infected by BtCoV.

Obtained sequence data were carefully checked for peaks and 327 bp for the RdRp region and 547 bp for the S protein region of each amplicon, except primer sequences, were selected to construct phylogenetic trees of nucleotide sequences. Five different sequences of BtCoVs were detected in RT-PCR products of the RdRp region and two sequences were detected from RT-PCR products of the S protein region, and they were named in accordance with the rules for nomenclature of influenza virus, that is, BtCoV host species/isolated place/isolation number/isolated year. The viral genomic information was deposited in DDBJ/EMBL/GenBank (accession numbers AB619638–AB619642 and AB644273–AB644274). The phylogenetic trees were constructed along with other available coronaviral sequences deposited in GenBank using the MEGA4 software [19] (Fig. 1a: RdRp region, b: S region). The sequences detected from *M. fuliginosus* in Japan were classified into group 1 (alpha) coronavirus of BtCoVs and were quite different from SARS-related BtCoVs. In the RdRp region, BtCoVs in Japan are closely related to BtCoV HKU7-1, which was isolated from *M. magnater* in Hong Kong in 2006 [18], with 95% of nucleotides identified. BtCoV HKU8-1, which was isolated from *M. pusillus* in Hong Kong in 2006 [18], was the second related strain, but fewer nucleotides were identified (80%) than in HKU7-1. In the S protein region, M.ful./Japan/03/2009 showed 92% identity relative to BtCoV Fujian/773/2005 (GenBank EF434379), which was isolated in the Fujian province in China in 2005 [20]. In contrast, M.ful./Japan/04/2010 showed about 70% similarity with the HKU8 strain and other group 1 BtCoVs (1a and 1b), which were isolated in Hong Kong from 2004 to 2005 [21]. Both of two isolated Japanese sequences belonged to the group 1 (alpha) coronaviruses, but the identity between them was only 64%. Therefore, this suggests that different BtCoV strains were present in the same cave. We could not compare nucleotide sequences of the S gene between Japanese BtCoV and HKU 7-1 because the sequence for HKU 7-1 was not available in GenBank.

Discussion

In this study, 45 intestinal specimens and 15 pools of fecal specimens were tested for BtCoV with specific primer sets for RdRp and S regions. The number of PCR-positive pools was high: 5/45 (11%) of intestinal and 11/15 (73%) fecal specimens were positive with the primer set for the RdRp region and 1/45 (2%) of intestinal and 6/15 (40%) fecal specimens were positive with the primer set for the S protein region. The specimens were collected from two places; one was a habitat of *M. fuligunosus*, which lives in the Kinki region of Japan, and more than 20,000 female bats gather in the cave during the breeding season [13]. This suggests that BtCoV is endemic at a high frequency in *M. fuliginosus* in the Kinki region of Japan. In addition, BtCoVs in Japan belong to the group 1 (alpha) coronaviruses, and phylogenetic analysis of the RdRp and S protein regions indicated that it is quite different from SARS-related CoV. In particular, S protein of coronavirus is a major determinant of receptor binding and virus–cell membrane fusion [22]. Therefore, the differences in S protein region sequence imply the possibility that Japanese BtCoVs have different infectivity to host species compared to other SARS-related BtCoVs. Moreover, the S protein sequences of M.ful./Japan/03/2009 and M.ful./Japan/04/2010 showed 64% identity between them. This also

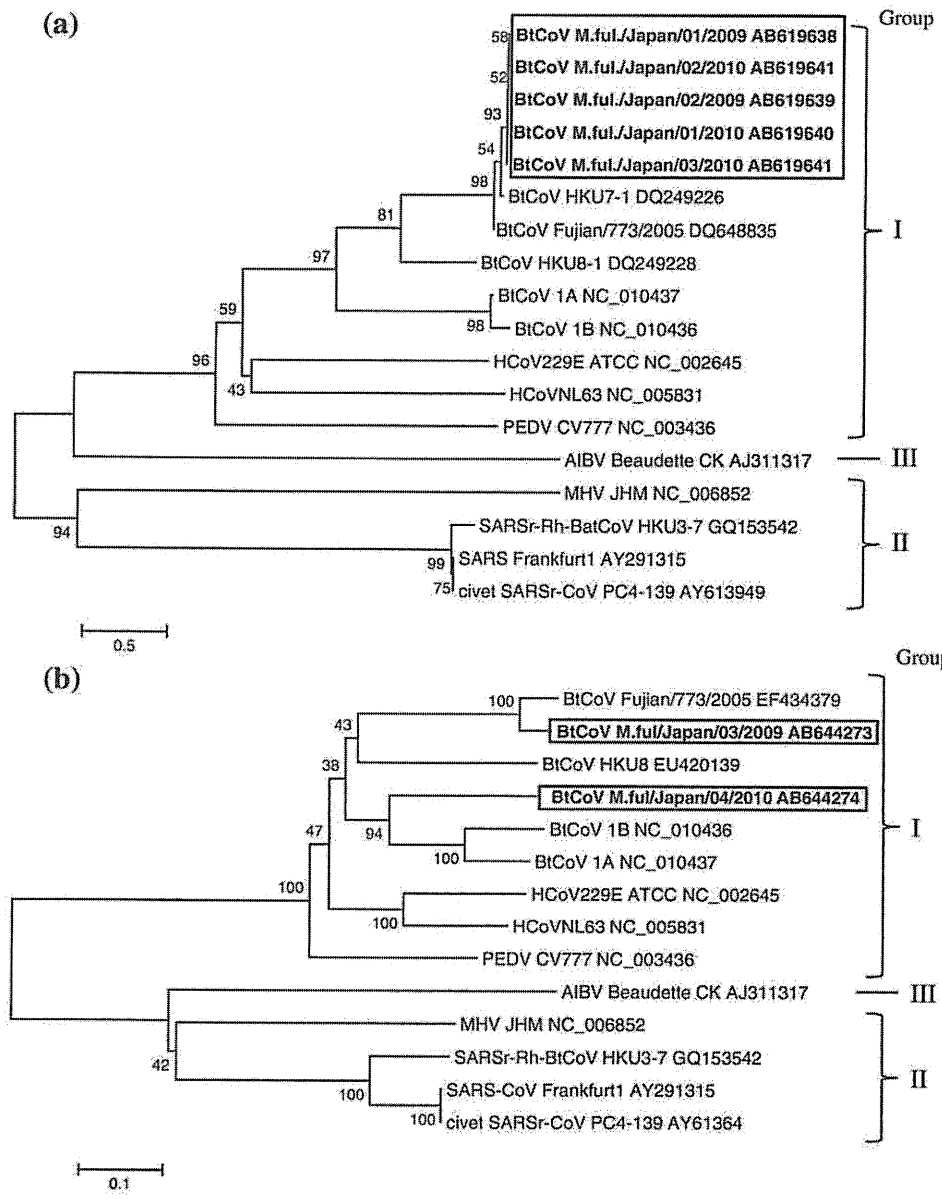

Fig. 1 The phylogenetic trees based on the nucleotide sequence of **a** RdRp and **b** S regions. The phylogenetic trees were constructed with the MEGA4 software using the bootstrap test command with the neighbor-joining method. The accession numbers of used coronaviral sequences were described in "Materials and methods" section

suggests that different strains of BtCoVs are endemic in the same place, like several different strains of human coronaviruses.

The nucleotide sequences of RdRp of BtCoV in Japan were similar (95%) to those of HKU7-1, which was isolated from *M. magnater* in Hong Kong, and the nucleotide sequences of S protein of Japanese BtCoVs also showed some similarity to other Hong Kong and Fujian strains. *Miniopterus* bats are a migratory species and one population has several habitats surrounding its central breeding cave [23]. The distance of bat migration is reported to be several 100 km [13, 24]. This implies the possibility that BtCoV-infected bats have been brought to Japan from Southeast China (or to there from Japan) through unknown routes. In addition, Rodrigues and Palmeirim [24] have reported that female bats nearly always return to their natal colony to give birth, whereas male bats sometimes go to other colonies, suggesting that BtCoV is transmitted between Japan and Hong Kong or Fujian by male bats. Similar cases have been reported for rabies virus. Rabies virus infection is associated with the migratory routes of Nathusius' pipistrelle (*Pipistrellus nathusii*) in France. It has also been reported that silver-haired bats (*Lasionycteris noctivagans*) migrate seasonally from Alaska, across Canada, to Texas, and rabies virus variants have been identified from several locations throughout the geographic range of these bats [25]. Therefore, BtCoV might be transmitted between Hong Kong and Japan as a result of bat migration.

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REVIEW *Public Health*

Alternative BSE Risk Assessment Methodology for Beef and Beef Offal Imported into Japan

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ABSTRACT. The Food Safety Commission (FSC) of Japan, established in July 2003, has its own initiative to conduct risk assessments on food stuffs known as "self-tasking assessment". Within this framework, the FSC decided to conduct a risk assessment of beef and beef offal imported into Japan from countries with no previous BSE reports; thus, a methodology was formed to suit to this purpose. This methodology was partly based on the previous assessments of Japanese domestic beef and beef imported from U.S.A./Canada, but some modifications were made. Other organizations' assessment methods, such as those used for BSE status assessment in live cattle by the OIE and EFSA's GBR, were also consulted. In this review, the authors introduce this alternative methodology, which reflects (1) the risk of live cattle in the assessed country including temporal risks of BSE invasion and domestic propagation, with the assessment results verified by surveillance data, and (2) the risk of beef and beef offal consisting of cumulative BSE risk by types of slaughtering and meat production processes implemented and the status of mechanically recovered meat production. Other possible influencing factors such as atypical BSE cases were also reviewed. The key characteristic of the current assessment is a combination of the time-sequential risk level of live cattle and qualitative risk level of meat production at present in an assessed country.

KEY WORDS: beef, BSE, importation, prion diseases, risk assessment.

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More than 20 years have passed since BSE was officially recognized in the U.K. Now, there is prominent evidence showing the efficacy of a real feed ban and the abolishment of using meat and bone meal (MBM) derived from mammals in feeds for mammals. The total number of BSE-positive cases in the world last year was less than that of one day when the BSE outbreak was at its peak in the U.K. from 1992 through 1993. However, the U.K. continued to spread the sources of BSE pathogens, such as live cattle and animal feeds, to two dozen countries, resulting in a cumulative number of more than 220 variant CJD patients in the world [9].

Currently, Japan imports beef and beef offal from the U.S.A. and Canada, two countries that have previously experienced BSE cases and for which the Food Safety Commission (FSC) in Japan has already assessed the BSE risks of their beef and beef offal. Besides these two countries, Japan also imports beef and beef offal from other countries where no BSE cases have been reported so far. However, some of these countries were categorized as Geographical BSE Risk (GBR) category III by the European Food Safety Agency (EFSA). According to EFSA's definition, countries are designated as GBR category III either because they are estimated to have a reasonably high possibility of having BSE cases that have not been detected or because they

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AUTHORS' NOTES: The authors, except for the first author, are listed alphabetically. This article is based on the discussion at a Prion Expert Committee meeting.

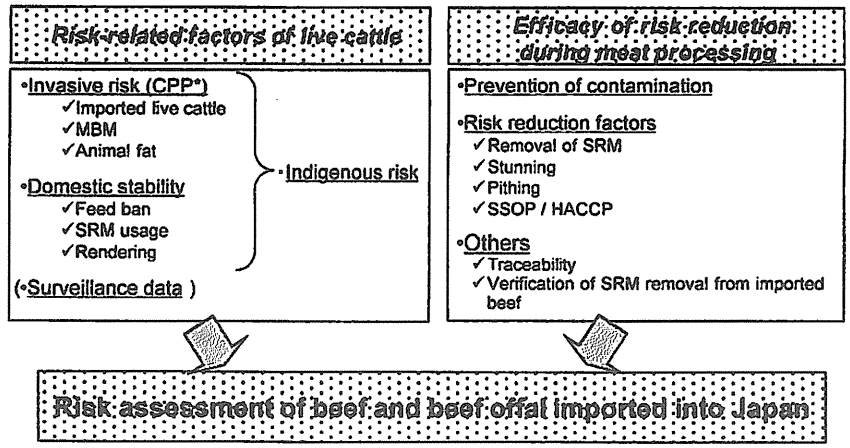

Fig. 1. Schematic presentation of the risk assessment methodology employed by the PEC for the current assessment. Briefly, the risk-related factors in live cattle (shown in the left box) were combined with the risk-reducing efficacy at meat processing facilities (shown in the right box) to gain the total risk assessment targeting beef and beef offal imported into Japan. *: Contamination probability points.

have had a few confirmed cases of BSE. Among exporters to Japan, there are also countries that have simply not been assessed by EFSA's GBR.

Japanese risk managers presently request importers of beef and beef offal from the above countries to submit official health certificates confirming that the cattle are of healthy origin and also ask that they refrain from importing specified risk materials (SRM). Although the health certificates are confirmed at quarantine stations, there are currently no measures to clarify the exclusion of SRM among beef products imported. There is also uncertainty over potential risks of imported beef and beef offal due to insufficient availability of data related to BSE prevalence and anti-BSE countermeasures in the above-mentioned countries.

The FSC in Japan conducts risk assessments at the request of risk managers, or alternatively, it can also conduct assessments on its own initiative, termed "self-tasking assessment". The process of hazard selection for self-tasking assessment is as follows. The Expert Committee for Planning collects information and screens the possible assessment subjects based on the degree of public concern in Japan, based on demands for information collection either due to increasing necessity for developing hazards or based on items that are heavily requested for assessment. Selected subjects are then discussed for potential assessment at the Commission's opinion exchange meetings, and finally, the FSC officially adopts the hazards of choice to be the next subject of self-tasking assessment.

Risk assessment of beef and beef offal imported into Japan was among the most requested items during public meetings and other occasions hosted by the FSC. Behind these requests, there seemed to be public concern over uncertainty about BSE risks in beef and beef products imported from countries other than the U.S.A. and Canada. With this situation, the FSC decided to conduct "risk assessment of beef and beef offal imported into Japan" as its self-tasking assessment.

The current assessment conducted by the Prion Expert Committee (PEC) of the FSC in Japan is based on the following concepts: (1) presently, the worldwide BSE prevalence is in the trend of decline; (2) this risk assessment is essentially different from the rest of the BSE-related risk assessments previously conducted by the FSC, in that the assessed countries are only those that have not previously reported BSE cases; (3) previous risk assessments of beef and beef products from the U.S.A. and Canada were conducted by comparing their risks with that of Japanese beef and beef products so that the assessment was based on the relativity; and (4) it was foreseen to be based on the data submitted by each assessed country on a voluntary basis. Subsequently, assuming that there may be certain limitations concerning data availability and submission, the PEC decided to largely conduct this assessment on a qualitative basis but to strive to make it as quantitative as possible.

It was with this background that the PEC firstly developed an alternative assessment method suited to the current situation and then carried out BSE risk assessment for imported beef and beef offal according to this method. In this review, the authors describe the structure and logic of this assessment method. A sample assessment result is provided at the end of this article to enhance readers' understanding.

PRINCIPLES OF THE CURRENT RISK ASSESSMENT

The methodology for the current risk assessment was developed based on the previously used models for risk assessments of Japanese domestic beef and for US/Canadian beef imported into Japan [5, 6]. OIE's risk assessment criteria for BSE status and the EFSA GBR method were also referred to [8, 11]. The PEC for the current assessment aimed

to deliver the overall conclusion as a science-based comprehensive assessment defined by time periods and based on a combination of the following risk aspects: (1) periodic BSE risk status among the cattle population of a country, which consisted of combined risks of BSE invasion by imported live cattle and MBM, and domestic stability (inversed risk of BSE propagation), of which the latter included implementation of a feed ban and establishment of preventive measures against cross-contamination, etc., and (2) present risks of beef and beef offal processing lines, i.e., risks based on types of slaughtering and meat production processes, etc.(Fig. 1).

The current assessment was conducted on a qualitative basis rather than a quantitative basis because of the limited data regarding BSE risks. In case the data were insufficient, assessment was based on the worst-case scenario. In addition, a few cases of atypical BSE, which is biologically and biochemically different from typical BSE, have been recently found in Europe, Japan and the U.S.A. among other countries. Those cases were distinguished from the classical type of BSE mainly by band patterns of PrP^{Sc} proteins demonstrated by Western blotting. The origin of atypical BSE is still unknown to this date, and information about BSE prion distribution in bovine tissue is scarce [2, 3].

Due to the above-mentioned situation, therefore, the current risk assessment was conducted with the assumption that (1) the first case of BSE occurred in the U.K. for an unknown reason, with BSE agents then being propagated through MBM recycling from BSE-infected cattle, and that (2) BSE infection was spread to other countries by exportation and utilization of BSE-infected live cattle and BSE-contaminated MBM for animal feeds.

PERIODIC BSE RISK STATUS AMONG THE CATTLE POPULATION OF AN ASSESSED COUNTRY

Assessment of invasive BSE risk: For the purpose of analysis in this section, the PEC defined a country in categories III or IV according to the EFSA GBR or a country with at least one BSE-positive case reported among its domestic cattle in the past as a "BSE risk country". Invasive BSE risk was assessed based on their records of live cattle, MBM and animal oil/fat importation from the BSE risk countries defined by this description.

The determined BSE risk countries were divided into the following subgroups by the level of BSE contamination as follows: the U.K., European countries with moderate contamination, European countries with low contamination, the U.S.A., Canada and others (Japan, Mexico, Chile, etc.).

Accordingly, each assessed country exporting beef and beef offal to Japan was requested to submit data regarding imports of live cattle and MBM from the BSE risk countries. Portugal had been categorized as a level IV country by the FESA GBR together with the U.K. and thus should not be grouped with other moderate-risk European countries. Nevertheless, such distinction was not made because no assessed country had a record showing importation from Portugal.

Submitted information was analyzed for possible use of the imported live cattle and MBM for animal feed production in the assessed country. In the case that the records submitted by the assessed country indicated any degree of possibility of live cattle and MBM imports from BSE risk countries having been used for animal feed, the degree of invasive BSE risk in the assessed country was estimated as a sum based on contamination probability points (CPP) of each BSE risk country. The assessment was based on a 5-year period, as this was considered to be the general term for BSE incubation.

Risks of animal oil and fat varied depending on the products' grades such as yellow grease, fancy tallow, etc., but their risks were generally regarded as low compared with that of live cattle or MBM. Thus, the information associated with animal oil/fat and their usages were taken into consideration only when importation of large quantity was recorded from BSE risk countries. Otherwise, the data were used as supplementary information.

Contamination probability points (CPP): All imported live cattle and MBM, in principle, have a potential to be used for animal feed manufacturing, but when a country could provide a feasible explanation for not utilizing any of the imported live cattle or MBM for animal feed, they could be determined as carrying no risk and thus excluded from consideration of risks.

When the track records of the imported live cattle and MBM in the assessed country showed any of the following destinies, they were regarded as adding no risk to the assessed country: (1) imported live cattle were already dead and disposed of by burial or incineration; (2) imported live cattle were still alive at the time of investigation, so they were excluded from potential use for animal feed manufacturing beforehand; and (3) imported live cattle and MBM were recorded to have been re-exported to other countries.

In this assessment, the PEC defined the invasive BSE risk as combined CPPs of imported live cattle and MBM. Its assessment was to be calculated based on the assumption that 1 ton of MBM was equivalent of 1 live bovine animal, as has been stated in the GBR by the Scientific Steering Committee (SSC) and EFSA [10].

The risks of imported live cattle and MBM from the BSE risk countries varied depending on the country and timing of importation. To reflect this variation, this assessment employed CPPs for live cattle and MBM of each BSE risk country. Records showed that the BSE prevalence in the U.K.'s live cattle was 5% at its peak period of 1988–1993; therefore, a CPP of 1 was set as the risk of importing 1 live bovine animal from the U.K. during this period. Thus, the CPPs of the U.K. were set as shown in Table 1 based on the values indicated by the SSC's GBR and years of complete feed ban implementation in the U.K. [10].

European countries except for the U.K. were divided into two categories, namely countries of "moderate contamination" and "low contamination" [11]. The CPPs for live cattle and MBM were set up based on the SSC's GBR and years of complete feed ban implementation in European countries [4, 10] (Table 2). Countries such as France, the Netherlands, Belgium, and Italy were likely countries to have re-exported MBM from the U.K. and thus were given a CPP of 0.1 until

Table 1. Periodic CPPs of live cattle and MBM from the U.K.

Live cattle		MBM (1 ton)	
1987 and years before	0.1		
1988–1993	1	1986–1990	1
1994–1997	0.1	1991–1993	0.1
1998–2005	0.01	1994–2005	0.01
2006 and years after	0.001	2006 and years after	0.001

the U.K. banned exportation of MBM (years of 1986–1996).

In the previous risk assessments done by the PEC of the FSC in Japan concerning U.S.A./Canadian beef imported into Japan, the surveillance-based BSE prevalence of U.S.A. and Canadian cattle were estimated to be 1 case and 5–6 cases per one million cattle in the U.S.A. and Canada, respectively [6]. Accordingly, the CPPs of live cattle and MBM in those two countries were set. The values are given for the periods defined by estimated year of birth among BSE-positive cattle (Table 2).

In the previous risk assessment done by the PEC of the FSC concerning U.S.A./Canadian beef imported into Japan, the surveillance-based BSE prevalence of Japanese cattle were estimated to be 5–6 cases per one million cattle. Birth years of BSE-positive cattle and the year of feed ban implementation were also taken into account to set the following CPPs for live cattle and MBM of Japan [6] (Table 2).

The CPPs for countries with no reported BSE cases could not be set by the above-mentioned BSE prevalence-based method. Since those countries were generally considered to have low BSE risks compared with countries with BSE-positive cases, CPPs were not determined for these countries. In the case that an assessed country imported a large quantity of live cattle and/or MBM from BSE-negative and GBR III countries, the information was taken into consideration as a supplementary factor for the assessment.

Assessment for invasive BSE risk: Based on the principles above, total invasive BSE risk (a sum of the invasion risks from imported live cattle and MBM) was estimated for each assessed country for a period of five years. The assessment was finally given in 5 levels, high, moderate, low, very low and negligible, as shown in Table 3.

Domestic Stability (inversed risk of BSE propagation of a country). Principles of domestic stability assessment: The essential countermeasures against BSE exposure/propagation consisted of (1) implementation of a feed ban, (2) control of SRM use, (3) optimization of rendering conditions and (4) establishment of preventive measures against cross-contamination for feed production.

Previous epidemiological analyses indicated that the most effective measure implemented in Europe was feed ban. Thus, an essential part of BSE exposure/propagation prevention was to abolish feeding of cattle with possibly BSE-contaminated MBM through animal feeds. It is in this context that a feed ban has been implemented in countries as a preventive measure against BSE. At the pragmatic level, the most effective way was to ban recycling of animal proteins regardless of animal types among mammals (ban from

Table 2. CPPs of live cattle and MBM from various countries

European countries with moderate contamination[a)]	
1986–2005	0.01
2006 and years after	0.001
European countries with low contamination[b)]	
1986–1990	0.001
1991–2005	0.01
2006 and years after	0.001
U.S.A.	
1993–	0.00002
Canada	
1989–	0.0001
Japan	
1992–2006	0.0001
2007 and years after	0.00001

a) France, Netherlands, Belgium, Italy, Ireland, Germany, Spain, Swiss, etc. b) Poland, Denmark, Austria, Czec Republic, Slovenia, etc.

Table 3. Total invasive BSE risk

Levels for risk of invasion	U.K. equivalent (N) [a)]
High	100≤N
Moderate	20≤N<100
Low	10≤N<20
Very low	5≤N<10
Negligible	0≤N<5

a) Calculated based on the assumption of 1 ton of MBM equals 1 live bovine animal.

mammals to mammals), followed by less but still effective measures such as a ban on protein recycling from mammals to ruminants and then from ruminants to ruminants.

Other measures that were also indicated as important for BSE control in these analyses included exclusion of SRM from rendering materials, optimization of rendering conditions (not less than 133°C for a minimum of 20 min at an absolute pressure of 3 bar), dedication of feed mills to a single species and production line separation.

It has been stated that 99% or more of infectivity in BSE-positive bovine animals is distributed to the bodily regions called specified risk material (SRM; e.g., brain, spinal cord, etc.) [4]. Removal of SRM from rendering materials was considered to be important, and the best way to realize this measure was implementation of a legally-bound feed ban that prohibited the use of SRM and fallen stock in animal feed. Even diversion of SRM use from feed production to human consumption was considered to provide a certain degree of protection against BSE exposure/propagation, when coupled with avoidance of fallen stock use in animal feed.

Rendering under proper conditions could provide an effective reduction in BSE infectivity. For example, heat treatment (126°C for 30 min) of a prion strain (301V strain) after passage using mice resulted in reduction of infectivity by