log1.9 (ID_{50}/g) or log 2.7 (ID_{50}/g). The scientific opinion of the EFSA is that heat treatment of BSE prion under certain conditions (133°C for a minimal of 20 min at bar 3) effectively reduces its infectivity by 1,000 times [12], although the same rendering conditions may not be as effective as indicated above when the subject is a mixture of SRM and bones originating from BSE-infected cattle. Drawn from this data was the suggestion that, although the heat treatment recommended by the OIE appeared to be effective in reducing risks to certain extent, other measures should be combined with this rendering policy to completely prevent BSE exposure/propagation.

A previous study reported that oral administration of 0.1, 0.01 and 0.001 g of BSE-infected cattle brain was capable of transmitting BSE at the rate of 7 in 15 cattle, 1 in 15 cattle and 1 in 15 cattle, respectively [13]. These data were consistent with the European field observation that even a trace amount of animal protein was enough to infect bovine animals through contaminated feeds. Therefore, simply washing of the processing lines in a feed factory was not sufficient to prevent cross-contamination; implementation of more advanced measures such as dedication of feed mills to a certain species of animals and line separation were required.

For assessment of domestic stability, the information submitted from each assessed country was firstly analyzed for the extent of feed ban implementation, and then other aspects such as use of SRM, rendering conditions and preventative measures against cross-contamination were evaluated.

The assessment also focused on the degree of legal obligation bound to each regulation. The data regarding compliance with those preventative regulations were also evaluated whenever the data were available.

Assessment of domestic stability: Based on the assessment principles described above, countries were firstly assessed for their domestic stability by rigidity of feed bans (e.g., abolishing the use of all mammalian products for mammalian feed, mammalian products for ruminant feed or ruminant products for ruminant feed regulations [①–④ in Fig. 2]). Secondly, regulation of SRM usage was considered (◎–○). In the case that SRM was used for feeds, rendering conditions and avoidance of cross-contamination were considered. Risk reduction measures such as rendering at 133°C for 20 min at 3 bars and/or prevention of cross-contamination at feed factories, e.g., isolating production lines or establishing exclusive feed production facilities were also taken into account (○ or △). Finally, the domestic stability of each country was assessed by categorizing them into one of 5 ranks, very stable, stable, middle, unstable and very unstable, based on a certain period defined by significant events such as regulatory modification.

Assessment of overall BSE risk in a cattle population, aided by verification with surveillance: Countries with high BSE propagation risks presumably bear a high risk of domestic BSE exposure/propagation upon entrance of BSE agents into the country. Thus, when the submitted data indicated a high risk of domestic BSE exposure/propagation (namely, combined risks of high invasive BSE risk and unstable domestic stability), this aspect was taken into consideration as an additional factor. In the case that an assessed country was categorized in a high exposure/propagation period at a certain time in the past, that country was assigned to a one-rank higher overall risk group for the next 5 years.

Surveillance was the essential method to scientifically verify the risk assessment output, and so surveillance data obtained from an assessed country was used for the results of the current assessment. The actual verification process was constructed based on the OIE point system (BSurvE method), as no other alternatives were available at that time.

RISK ASSESSMENT OF THE PROCESS OF BEEF AND BEEF OFFAL PRODUCTION

Ensuring the removal of SRM can remarkably reduce the risk of variant Creutzfeldt-Jakob disease (vCJD) in humans. Therefore, this measure is currently at the center of preventive policies regarding protection of human and cattle health from BSE. The current risk assessment for beef and beef offal firstly evaluated the extent of "SRM removal" done by each country, followed by combined assessment of items such as "inspection at slaughterhouses" and "stunning/pithing" to evaluate the risk-reducing efficiency of meat processing lines.

SRM removal: In a BSE-positive bovine animal, 99% or more of its infectivity is attributed to SRM [9]. Thus, exclusion of these materials from the food chain ensures reduction in most of the vCJD-associated risks in humans. To reflect these SRM-related aspects, the current assessment also took into consideration factors such as implementation of SRM removal or any other measures in preventing beef and beef offal from being contaminated by BSE agents. This part of the assessment was ultimately designed to determine whether cross-contamination preventive measures and efficacy verification systems for them had been established in each country.

The definition of SRM adopted in this assessment was from the OIE's SRM definition for a "controlled risk country" based on the following reasons: (1) the currently assessed countries all had no BSE-positive reports; (2) there were, however, some countries that were categorized into GBR III of the EFSA; and (3) the definition and handling of SRM varied among the assessed countries. When the SRM definition of the assessed country was acknowledged to be largely different from that of the OIE, the assessment was conducted in a manner separate from these general principles and handled on a case-by-case manner.

Inspection, stunning and pithing at slaughterhouses: Elimination of high risk cattle such as downer cows is an important protocol in protecting human health from BSE risks, and for this reason, the OIE code requires proper antemortem inspection before slaughtering [9]. However, it is also known that clinical observation for possible symptoms alone is not enough to distinguish BSE-infected cattle from other diseases. Therefore, both the provisions of (1) effective elimination of downer cows at the antemortem inspection and (2) BSE testing at slaughterhouses were evaluated in the current assessment.

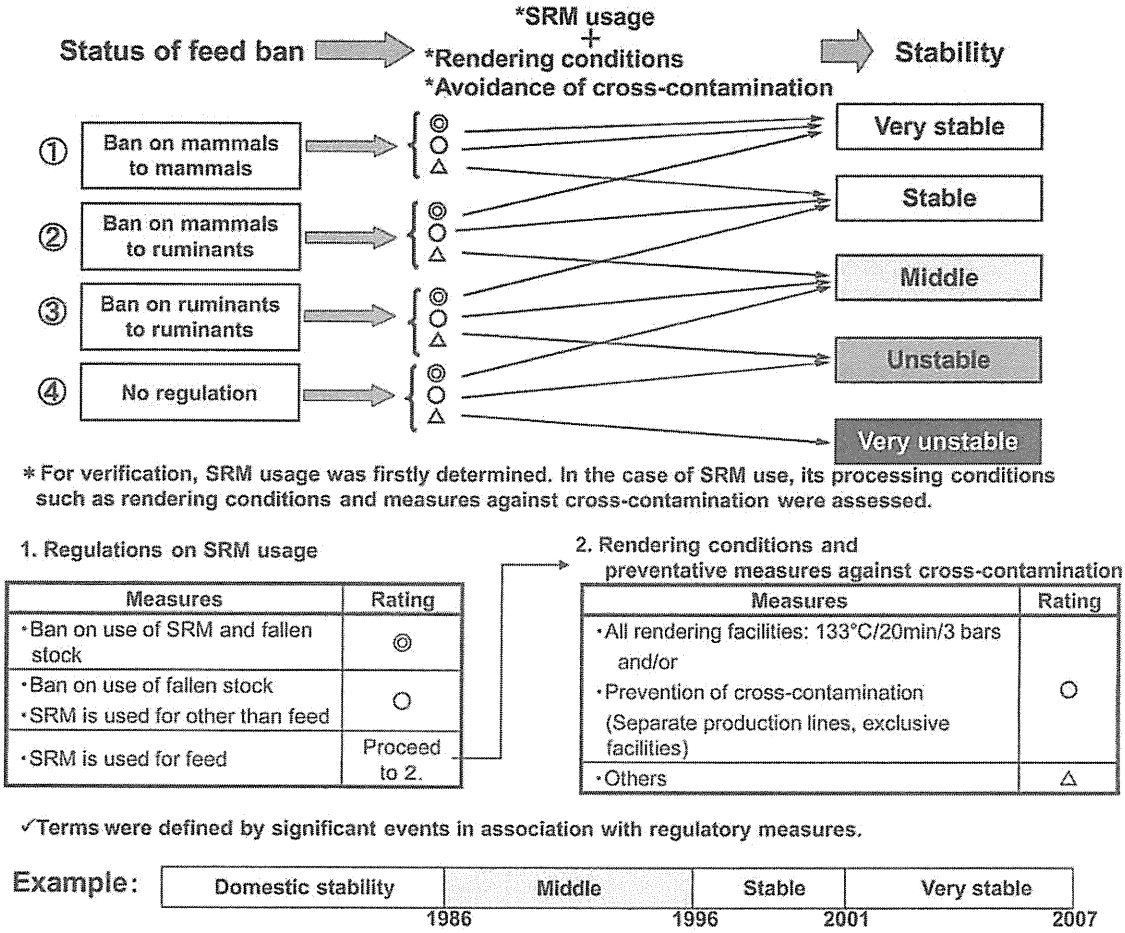


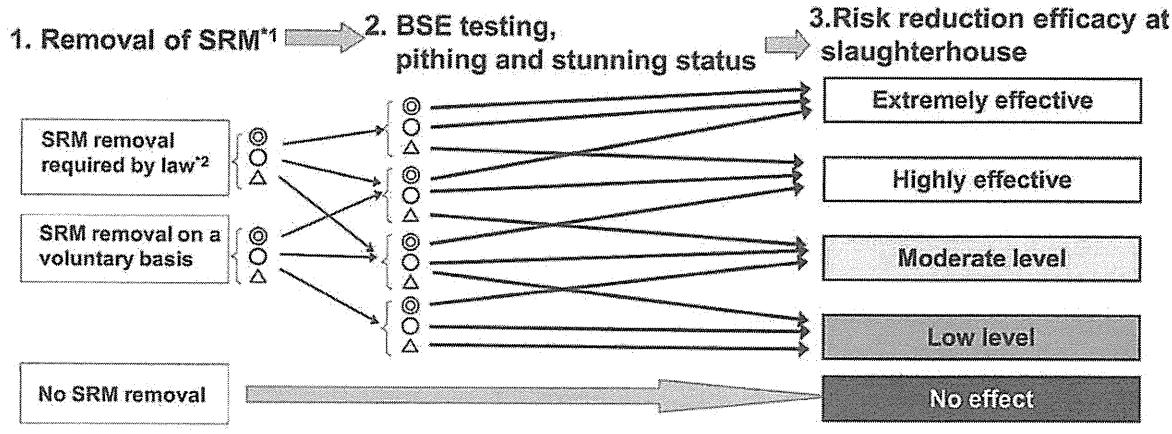
Fig. 2. Assessment of domestic stability. Each country was evaluated by the range of subjected animals and rigidity of the feed ban, followed by SRM-related conditions, to be assigned to one of the 5 ranks indicating degree of domestic stability. The rigidity of feed ban was rated based on the strengths of SRM-handling measures (the two lower boxes). At the bottom, an example of domestic stability is shown per terms defined by significant regulatory changes.

Pithing of animals at the slaughterhouse is linked to an increased risk of BSE contamination via brain and spinal tissue spillage from the stunning hole onto the processed meat and slaughtering facilities. It also increases the possibility of high-risk tissue (brain and spinal cord) leakage into the blood stream. Likewise, a stunning method with intracranial air/gas pressuring may also bring about a similar manner of contamination [1]. Therefore, the current assessment took into consideration slaughtering processes such as implementation of pithing or air/gas injection stunning in each assessed country.

Others (mechanically recovered meat, MRM, etc.): MRM including meat from advanced meat recovery (AMR) is meat obtained from bones by secondary recovery using mechanical techniques after primary removal of the major meat blocks. This method carries a certain risk of SRM contamination. Thus, the same assessment method as for primary beef meat blocks cannot be adopted for MRM in evaluation of the risk-reducing efficacy of BSE measures. Accordingly, the Commission requested the assessed countries known for MRM production to submit additional information regarding the SRM definition, measures and MRM exportation to Japan, and then these data were assessed separately from general beef and beef offal exports.

The total number of livestock and their traceability were also requested as supplementary data because they are related to the sensitivity and precision of antemortem inspection at the slaughterhouse or estimation of animal age in months.

Overall assessment of risk-reducing measures at meat processing lines: The process of assessment for efficacy of risk reduction during meat processing is concisely demonstrated in Fig. 3, aided by the explanation as follows. The efficacy of risk reduction during meat processing was assessed firstly by the degree of SRM removal. In the case that SRM removal was conducted in accordance with regulations, the risk reduction level was regarded as the highest, followed by voluntary removal; no regulation was the worst grade. Secondly, the actual measures used for SRM removal were



*1 Judgment was based on the conditions of SRM removal and procedures at slaughterhouses (If available, data of actual compliance was also taken into consideration.)
*2 Removal by domestic regulation, or by additional conditions required for exportation to Japan

1. SRM removal

Measures	Rating
① Confirmation by meat inspector ② Carcasses washed by high pressure water ③ Washing of the saw between carcasses ④ SSOP and HACCP regulations 3 or 4 of the above measures implemented	◎
2 of the above measures implemented	○
Others	△

2. BSE testing, stunning and pithing status

Measures	Rating
・Health inspection to eliminate downers and BSE testing at slaughterhouses and ・Ban on air pressure stunning and pithing Both measures implemented	◎
One measure implemented	○
Others	△

Fig. 3. Flow chart of the assessment for efficacy of risk reduction at slaughterhouses. Each county was firstly reviewed for the status of SRM removal, followed by the rating of other risk-avoiding measures, to determine its risk reduction efficacy.

verified based on the actual conditions for such things as meat inspector's process confirmation, washing of carcasses with high pressure water to assure the complete removal of central nervous tissues, washing of the saws between individual carcasses and regulation by a Sanitation Standard Operating Procedure (SSOP) and Hazard Analysis and Critical Control Points (HACCP). If 3 or 4 of the above measures were conducted, the risk reduction efficacy was given a one-rank promotion when proceeding to the next assessment point (◎). The second verification point for risk reduction was the combination of BSE testing and absence of pithing/air stunning. If health inspections to eliminate downers and BSE testing were conducted in the slaughterhouse together with bans for both air pressure stunning and pithing, the efficacy of risk reduction was promoted one rank (◎). If one of the above measures was conducted, the efficacy of risk reduction stayed at the same level (○);otherwise, the country was demoted one rank. Based on the above-mentioned principles, each assessed country was categorized according to 5 grades for the efficacy of its risk-reducing measures, namely no effect, low level, moderate level, highly effective and extremely effective (Fig. 3).

Since the current assessment subjected only the meat products imported by Japan, the scope of evaluation was focused on the criteria for beef product preparation and BSE-preventive measures intended for each country's exports to Japan. Presently, all the beef and beef offal importers in Japan are requested to voluntarily refrain from importing SRM from any foreign countries. Some exporting countries even have their own specific regulations on exclusion of SRM from beef and beef offal exports to Japan under the Animal Health Requirement requested from Japan. Therefore, these risk control measures were also taken into the assessment along with the information obtained from each country upon the Commission's request through a questionnaire.

RISK OF MECHANICALLY RECOVERED MEAT (MRM)

Among the countries that have submitted replies to Japan's inquiry, two countries reported domestic production of MRM, with one country having exported 81.6 kg of MRM (head parts not included in raw materials) to Japan in 2008, while the other had no such record.

The key structure of the current risk assessment was the evaluation of imported beef and beef offal to Japan, which consists of a combination of multiple aspects such as risk of live cattle and risk-reducing measures at slaughterhouse

and meat processing lines in fabrication plants. Accordingly, any commodities that do not fall into these categories, such as MRM, should be considered separately. There are so far no grounds to suggest that MRM contamination is not connected with SRM through meat processing lines. Thus, at least MRM from those countries that have potentially had exposure to and/or propagation of BSE in the immediate past according to the data collected should be regarded as carrying certain risks. However, MRM from countries that are regarded as having a negligible possibility of BSE exposure/propagation may be considered as carrying negligible risks, provided the precondition of the current assessment, namely classical BSE originating from the U.K., is appropriately met.

Recently, there have been a few cases of irregular forms of BSE (atypical BSE) reported apart from classical BSE in Europe, Japan and the U.S.A. These reports of atypical BSE indicated variation in molecular sizes of abnormal prion proteins (PrPSc) among cases, and eventually two major sizes of proteins were designated as the H and L types.

Most of the atypical BSE cases were found in aged cattle over 8 years old, but a remarkable exception exists in Japan, where a steer only 23 months old was reported to have been infected with atypical BSE (the 8th BSE case in Japan). When this exception was excluded, the detection ages of atypical BSE cases ranged from 6.3 to 18 years old. The average detection ages for the H and L types were 11.8 and 11.6 years old, respectively [3].

To the best of the authors' knowledge, there have been about 40 cases of atypical BSE reported worldwide, yet the OIE does not require distinction between classical and atypical BSE cases in member countries for their reports, while the EFSA only recently referred to case reporting by classical/atypical recognition in its 2009 scientific opinion. These situations seem to further obscure the clear number of atypical BSE cases occurring in the world.

The origin of atypical BSE has not yet been determined. According to EFSA's scientific opinion published in 2008, all the cases of atypical BSE were reported with birth dates before the real feed ban in January 2001 in Europe. Therefore, the possibility of these atypical cases being attributed to contaminated feeds, just as in classical BSE, cannot be completely denied. On the other hand, data of atypical BSE cases (both the H and L types) in France did not show any reasonable correlation between birth year and frequency of occurrence, as was indicated in classical BSE cases, thus raising the possible interpretation of atypical BSE being sporadic isolated cases of prion disease [3].

Based on the data accumulated in France, the frequencies of atypical BSE cases per 1 million tested adult cattle were estimated to be 0.41 and 0.35 cases for the H and L types, respectively (1.9 and 1.7 cases for the H and L-types, respectively, when limiting the sampling to tested cattle over 8 years old). In Japan, a total of 10 million cattle including fallen stock and slaughtered cattle were tested for BSE, and the results showed no positive cases of the H type and 2 positive cases (case 8, a 23-month-old steer; case 24, a 169-month-old Japanese black cow) of the L type of atypical BSE. These data indicate that Japan has prevalence frequencies of 0 and 0.2 cases of the H and L types of atypical BSE per 1 million cattle including tested fallen stock and slaughtered cattle (zero and approximately 1.5 cases of the H and L types respectively, when limiting the sampling to tested slaughtered cattle over 8 years old).

Atypical BSE of both the H and L types was confirmed to be transmissible by intracerebral inoculation in transgenic mice expressing alleles of bovine or ovine PrP genes and of inbred mice. However, for transgenic mice expressing human prion protein, the L type but not the H type could be transmitted according to the previously published reports (recently, it was reported that H type also transmissible to the humanized transgenic mice). There have also been reports of glycosylation pattern transformation from L-type BASE3 PrP^{Sc}-like type to more of the classical BSE PrP^{Sc} type. This phenomenon was observed during passage using inbred and transgenic TgVR2 mice. As for the atypical cases of BSE confirmed in Japan, the 24th case of BSE was determined to have had the atypical L type at the detection age of 169 months old, and its sample was successfully transmitted to transgenic mice expressing bovine prion protein. However, transmission of a sample from the other case of atypical L-type BSE confirmed in Japan (the 8th case; detected at the age of 23-month-old) was reported to be unsuccessful in transgenic mice expressing bovine prion protein. The reason for this inconsistency is not clear at this time, although the possible presence of a limitation in the amount of prion protein accumulated in the subject's brain sample or that the inoculated volume was too low to reach the detection limit cannot be excluded.

A recent report has shown that the atypical L type of BSE has a higher degree of potential for pathogenicity than that its classical counterpart because incubation periods are shorter in atypical BSE transmitted to transgenic mice expressing human prion protein, suggesting that atypical BSE possibly has a higher degree of pathogenicity when compared to its classical counterpart [7].

In contrast to classical BSE, the systemic distribution of abnormal prion protein in atypical BSE cases is barely known. Therefore, it is unclear whether the brainstem is truly the optimal part for sampling and testing in H/L type detection. Likewise, information regarding the infectivity distribution of atypical BSE is scarce in bovine peripheral tissues and body fluid. All together, the lack of essential data hinders, to a certain extent, evaluation of the relative risk-reducing effects of various SRM removal measures for cattle.

Based on the currently available data concerning the potential risks for humans of atypical BSE and prevalence of atypical BSE, it may be too extreme to deny the risk of MRM, especially in MRM derived from aged cattle. However, the degree of influence of the presence of atypical BSE on our concept of the MRM risk will be limited to a low level under the circumstances with presently available knowledge and our discussion. In the meantime, one must also be reminded of the fact that only a limited amount of data is currently available concerning atypical BSE. A proper amount of dis-

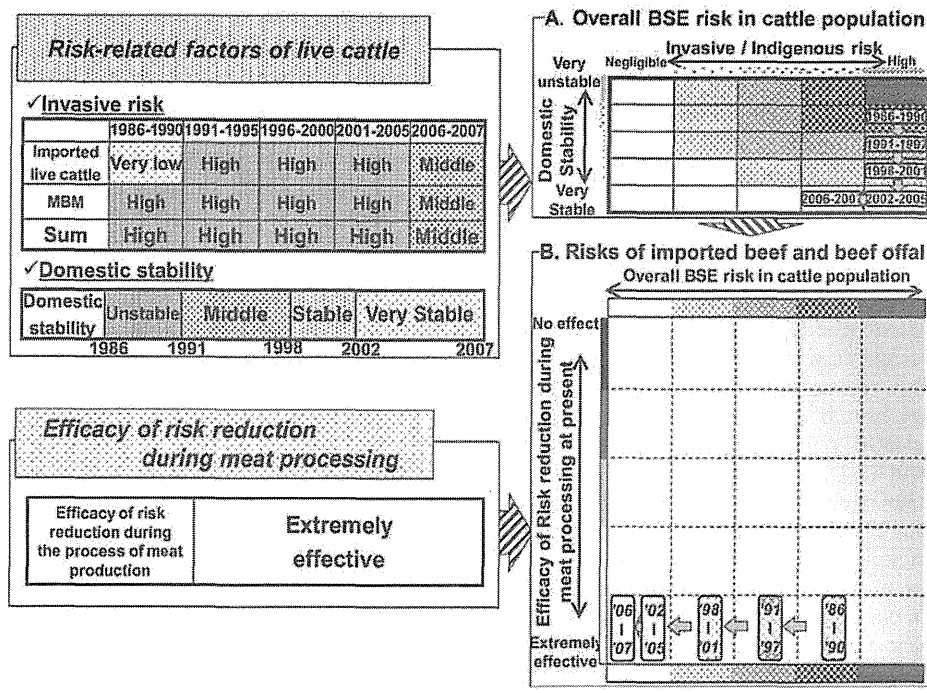


Fig. 4. Overall assessment results in a schematic view. A: The shift of overall BSE risks among age cohorts. The total invasive risks (the sum of imported live cattle and MBM risks; vertical axis) were plotted against domestic stability ranks (horizontal axis) in 2-D rank plot A. Note that the horizontal axis partially refers to the indigenous risk (domestic BSE propagation), which is also a reflection of invasive risk and domestic stability. Therefore, the results, shown in rank plot A, indicate the shift of BSE infection risks among the birth cohorts. B: The age-specific BSE risks in rank plot A and the efficacy of risk reduction during meat processing were combined. The outcome, 2-D rank plot B, was used to supplement the assessment process to get the final conclusion for each assessed country.

cretion should be used when interpreting these data to avoid unnecessary confusion. Further research and accumulation of data will bring additional insight into the mechanism, pathogenicity and transmission potential of atypical BSE, for which further assessment may become necessary in the future.

RISK ASSESSMENT OF BEEF AND BEEF OFFAL IMPORTED INTO JAPAN

To gain the final result of this assessment, the periodic BSE risk status of a country (the sum of invasive BSE risk and domestic stability) and efficacy of present BSE risk-reducing measures at meat processing lines were combined and used as an indicator of comprehensive likelihood of BSE prion contamination in beef and beef offal imported into Japan. Surveillance data were used to verify reliability of the assessment. Finally, a summary of each country was expressed in schematic figures (an example is shown in Fig. 4).

In Fig. 4, a model country's invasive risk was ranked as high (from 1986–2005) but was reduced to the middle level from 2006 onwards. The efficacy of feed ban (domestic stability) was unstable during 1986–1989 but improved to the middle level (1990–1996), to the stable level (1997–2000) and then to the very stable level (2001-until now). Current risk reduction efficacy at meat processing lines, determined by factors such as the definition of SRM, compulsory removal of SRM by law, and HACCP/SSOP procedures were good and verified and were therefore rated as ◎. BSE testing at slaughterhouse (>30 months), proper slaughtering procedures such as avoidance of air stunning and pithing were verified as ◎. All together, the overall risk reduction was extremely effective.

The final assessment for this model country was as follows: the domestic BSE exposure/propagation risk was low, and risk reduction at meat processing lines was extremely effective; therefore, the risk of BSE contamination of beef and beef offal imported from this assessed country was considered to be negligible.

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RESEARCH ARTICLE Open Access

Analysis of the humoral immune responses among cynomolgus macaque naturally infected with Reston virus during the 1996 outbreak in the Philippines

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Abstract

Background: Ebolaviruses induce lethal viral hemorrhagic fevers (VHFs) in humans and non-human primates, with the exceptions of Reston virus (RESTV), which is not pathogenic for humans. In human VHF cases, extensive analyses of the humoral immune responses in survivors and non-survivors have shown that the IgG responses to nucleoprotein (NP) and other viral proteins are associated with asymptomatic and survival outcomes, and that the neutralizing antibody responses targeting ebolaviruses glycoprotein ($GP_{1,2}$) are the major indicator of protective immunity. On the other hand, the immune responses in non-human primates, especially naturally infected ones, have not yet been elucidated in detail, and the significance of the antibody responses against NP and $GP_{1,2}$ in RESTV-infected cynomolgus macaques is still unclear. In this study, we analyzed the humoral immune responses of cynomolgus macaque by using serum specimens obtained from the RESTV epizootic in 1996 in the Philippines to expand our knowledge on the immune responses in naturally RESTV-infected non-human primates.

Results: The antibody responses were analyzed using IgG-ELISA, an indirect immunofluorescent antibody assay (IFA), and a pseudotyped VSV-based neutralizing (NT) assay. Antigen-capture (Ag)-ELISA was also performed to detect viral antigens in the serum specimens. We found that the anti-$GP_{1,2}$ responses, but not the anti-NP responses, closely were correlated with the neutralization responses, as well as the clearance of viremia in the sera of the RESTV-infected cynomolgus macaques. Additionally, by analyzing the cytokine/chemokine concentrations of these serum specimens, we found high concentrations of proinflammatory cytokines/chemokines, such as IFNγ, IL8, IL-12, and MIP1α, in the convalescent phase sera.

Conclusions: These results imply that both the antibody response to $GP_{1,2}$ and the proinflammatory innate responses play significant roles in the recovery from RESTV infection in cynomolgus macaques.

Keywords: Ebola, Ebolavirus, Reston virus, Reston ebolavirus, Filovirus, Zoonosis, Humoral immune response, Cynomolgus macaque, Cytokine, Antibody

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Background

The family *Filoviridae* includes three genera, Ebolavirus, Marburgvirus, and Cuevavirus. The genus Ebolavirus currently has five members: Bundibugyo virus (BDBV), Ebola virus (EBOV), Reston virus (RESTV), Sudan virus (SUDV), and Taï Forest virus [1]. Filoviruses induce lethal viral hemorrhagic fevers (VHFs) in both humans and non-human primates, while RESTV infection in humans is probably subclinical, yet it also causes highly lethal VHF in macaques [2,3]. RESTV epizootics among cynomolgus macaques emerged in 1989, 1990, 1992, and 1996. In all of these epizootics, the cynomolgus macaques originated in a single primate breeding facility in the Philippines [4]. Although the natural reservoir of RESTV remains unknown, RESTV was isolated from pigs in the Philippines, in addition to porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type-2 in 2008 [5]. Considering the social impact of ebolaviruses, it is important to understand the endemic and epizootic status of RESTV in the Philippines.

In this study, we investigated the antibody responses of cynomolgus macaques that could be dead-end hosts for RESTV. Using serum specimens collected from cynomolgus macaques during a RESTV outbreak in the Philippines in 1996, we attempted to elucidate the significance of neutralizing antibodies to RESTV in viral clearance. We have previously established an enzyme-linked immunosorbent assay (ELISA) and an indirect immunofluorescent antibody assay (IFA) specific for RESTV nucleoprotein (NP) [6-8]. These assays are useful tools for investigating the signs of RESTV infection in cynomolgus macaques. In human cases, antibody responses against ebolaviruses have been analyzed extensively: IgG responses to NP and other structural proteins (e.g., VP40 and VP35) have been shown to correlate with asymptomatic and surviving cases, and neutralizing antibody responses targeting the ebolaviruses glycoprotein ($GP_{1,2}$) appear to be the major indicator of protective immunity [9].

On the other hand, proinflammatory cytokines/chemokines are known to play a major role in the pathogenesis of ebolaviruses infections in various species. Previous studies have shown an uncontrolled secretion of proinflammatory cytokines/chemokines to contribute to a fatal outcome in EBOV-infected humans [10] and cynomolgus macaques [11]. Strong proinflammatory cytokine/chemokine responses are also observed in convalescent or asymptomatic cases [12,13]. In RESTV-infected cynomolgus macaques, high viremia has been shown to induce the secretion of proinflammatory cytokines/chemokines [14]. However, there have so far only been a limited number of studies on the impact of proinflammatory cytokine/chemokine responses in the convalescent phase of RESTV infection.

In this study, we grouped the cynomolgus macaque samples based on the presence of RESTV NP-antigen in sera and analyzed the antibody reactions and cytokine/chemokine inductions to evaluate the presence of neutralizing antibody to RESTV. We found that the anti-$GP_{1,2}$ responses, but not the anti-NP responses, were closely correlated with the neutralization antibody responses, as well as the clearance of viremia, in the sera of RESTV-infected cynomolgus macaques. Additionally, a high concentration of proinflammatory cytokines/chemokines was detected in the convalescent phase specimens. These data suggest that both the anti-$GP_{1,2}$ responses and proinflammatory cytokines/chemokines play significant roles in the recovery from RESTV infection in cynomolgus macaques.

Results

RESTV NP-and $GP_{1,2}$-specific antibodies, neutralizing antibody responses, and the viral antigens in the cynomolgus macaque sera from the 1996 RESTV epizootic

Twenty-seven serum samples derived from cynomolgus macaques that were either found already dead or had been euthanized at the facility were available. The presence of RESTV NP antigens was evaluated by antigen-capture ELISA [15] or immunohistochemistry [3], while that of anti-RESTV NP IgG was evaluated using IgG ELISA and IFA methods [6-8]. RESTV NP antigens were detected in the liver in # 2182, 2612, 2615, 2669, 2739, 2921, 2644 and 2728, while RESTV NP was detected by antigen capture ELISA in the sera of #2182, 2612, 2408, 2615, 2669, 2739, 2921, 2721 and 2972. We therefore assumed that these cynomolgus macaques had suffered from the acute viremic phase of the disease. Seventeen of the 27 samples (#2408, 2615, 2669, 2739, 2921, 2728, 2180, 2181, 2189, 2190, 2191, 2195, 2404, 2693, 2696, 2713 and 2194) were positive for anti-NP IgG in IgG ELISA, while these samples all reacted in IFA. On the other hand, two samples (#2644 and 2719) were only positive in IFA. We considered the samples as anti-NP IgG-positive when either ELISA or IFA showed positive reaction. As a result, a total of 19 samples had anti-NP IgG. Cynomolgus macaques with anti-NP IgG consisted of NP antigen-positive (#2408, 2615, 2669, 2739, 2921, 2644 and 2728) and NP antigen-negative groups (#2180, 2181, 2189, 2190, 2191, 2195, 2404, 2693, 2696 and 2713).

In order to examine whether the sera contained anti-$GP_{1,2}$ antibodies, we employed a $GP_{1,2}$-specific ELISA and IFA [16]. RESTV $GP_{1,2}\Delta TM$ prepared by a baculovirus expression system and RESTV $GP_{1,2}$-expressing HeLa cells were used as antigens for $GP_{1,2}$-specific ELISA and IFA, respectively. Anti-RESTV $GP_{1,2}$ IgG were detected in 10 (#2180, 2181, 2189, 2190, 2191, 2195, 2404, 2693, 2696 and 2713) out of the 27 serum samples according to ELISA (37%), whereas the

remaining 17 samples (63%) showed negative reactions. Nine serum samples positive for $GP_{1,2}$ antibodies in the IgG-ELISA also showed positive reactions in the IFA, while one serum sample (#2194) was only positive in the IFA. Serum samples showing positive reactions in either the $GP_{1,2}$-specific IgG-ELISA or IFA were considered to be anti-$GP_{1,2}$ positive (11/27, 41%, Table 1). Interestingly, the sera derived from cynomolgus macaques in the acute viremic phase did not contain any detectable anti-$GP_{1,2}$ IgG, although they often contained anti-NP IgG.

We next attempted to detect the neutralization (NT) antibody response in the sera of RESTV-infected cynomolgus macaques. The VSV pseudotype RESTV $GP_{1,2}$ (VSV-RESTV-$GP_{1,2}$/GFP) was used for the NT assay [17]. Twelve serum samples (12/27, 44%) neutralized the VSV-RESTV-$GP_{1,2}$/GFP infection, with NT titers ranging from 80 and 640 (#2721, 2972, 2180, 2181, 2189, 2190, 2191, 2195, 2404, 2693, 2696 and 2713) (Table 1). The anti-$GP_{1,2}$ IgG were not detectable in #2721 and #2972 by IgG ELISA, while those samples both had a neutralizing activity. These two specimens showed a positive response for viral antigen in the Ag-capture ELISA and were thus considered to be collected in an early seroconversion phase.

All cynomolgus macaques at the facility were euthanatized regardless of clinical status and there was a possibility that some of the cynomolgus macaques had combined infection with simian hemorrhagic fever virus (SHFV) in the animal facility [18]. Therefore, immune responses against RESTV did not always reflect the

Table 1 Antibody responses and viremic status of Reston virus-infected cynomolgus macaques

Case ID	Anti-NP IgG		Anti-$GP_{1,2}$ IgG		NT	Ag-ELISA		Overall status	Dead or **euthanized
	ELISA	IFA	ELISA	IFA		liver	serum		
2182	-	<80	-	<80	-	+	+	Ag + NT -	euthanized
2612	-	<80	-	<80	-	+	+	Ag + NT -	euthanized
2408	+	10240	-	<80	-	*ND	+	Ag + NT -	#NR
2615	+	2560	-	<80	-	+	+	Ag + NT -	euthanized
2669	+	2560	-	<80	-	+	+	Ag + NT -	euthanized
2739	+	1280	-	<80	-	+	+	Ag + NT -	euthanized
2921	+	1280	-	<80	-	+	+	Ag + NT -	euthanized
2644	-	80	-	<80	-	+	ND	Ag + NT -	euthanized
2728	+	1280	-	<80	-	+	ND	Ag + NT -	euthanized
2721	-	<80	-	<80	80	+	+	Ag + NT +	euthanized
2972	-	<80	-	<80	160	+	+	Ag + NT +	dead
2180	+	1280	+	320	80	-	-	Ag - NT +	dead
2181	+	80	+	80	320	-	-	Ag - NT +	dead
2189	+	1280	+	320	320	-	-	Ag - NT +	NR
2190	+	160	+	640	640	-	-	Ag - NT +	NR
2191	+	640	+	320	320	-	-	Ag - NT +	NR
2195	+	2560	+	640	320	-	-	Ag - NT +	NR
2404	+	1280	+	160	160	-	-	Ag - NT +	dead
2693	+	160	+	<80	320	-	-	Ag - NT +	euthanized
2696	+	2560	+	160	320	-	-	Ag - NT +	euthanized
2713	+	5120	+	320	160	-	-	Ag - NT +	euthanized
2719	-	80	-	<80	-	ND		ND	NR
832	-	<80	-	<80	-	ND		ND	NR
888	-	<80	-	<80	-	ND		ND	NR
1134	-	<80	-	<80	-	ND		ND	NR
2636	-	<80	-	<80	-	ND		ND	euthanized
2194	+	5120	-	80	-	ND		ND	dead
No. of positive samples	17/27	19/27	10/27	10/27	12/27 (44%)	11/21 (52%)			
	19/27 (70%)		11/27 (41%)						

*ND: not determined, **euthanized: monkeys were euthanized regardless of clinical manifestation. #NR: not recorded, *Ag* +: antigen positive, *Ag* -: antigen negative, *NT*+: neutralization antibody positive, *NT*-: neutralization antibody negative. The specimens with case IDs written in italics were considered in acute phase. The specimens with case IDs written in bold were considered in convalescent phase.

clinical manifestation. For these reasons, we defined "convalescent" or "non-convalescent" based only upon serological findings.

It is noteworthy that, among the serum samples that were positive for viral antigen and negative for the NT antibody (Ag +, NT -), all nine serum samples were negative for anti-$GP_{1,2}$ antibodies, whereas only two samples were negative for anti-NP antibodies. On the other hand, all of the ten serum samples that were negative for viral antigen and positive for the NT antibody (Ag -, NT +) were positive for both anti-$GP_{1,2}$ and anti-NP antibodies. This finding indicates that the anti-$GP_{1,2}$ antibody may therefore increase in cynomolgus macaques in the convalescent phase, while anti-$GP_{1,2}$ antibody is rarely detectable in the acute viremic phase of infection.

Multiplex assay for cytokines and chemokines in the cynomolgus macaque sera

Ebola virus infection triggers the expression of several proinflammatory cytokines/chemokines [11,19,20]. To examine whether the convalescence from the RESTV infection correlates with the circulating proinflammatory cytokines/chemokines, eleven RESTV-infected cynomolgus macaque serum samples were subjected to a multiplex cytokine analysis. Since we do not know when the infection occurred for each cynomolgus macaque, it is still unclear whether the sera represented an early or late stage of infection. We used seven convalescent phase sera (Ag-, NT +: #2404, 2181, 2189, 2693, 2696, 2713, 2180), and four acute viremic phase sera (Ag +, NT -: #2182, 2612, 2739, 2921). Among the 27 serum samples, only these 11 serum samples were available for multiplex assay. Since the sera were heat-inactivated at 56°C for 30 min prior to being subjected to the multiplex analysis, some cytokines, such as GM-CSF and IL-2, which were previously shown to be elevated in some RESTV infected cynomolgus macaques [14], could not be measured in the assay.

We found that concentrations of several proinflammatory cytokines/chemokines (e.g., IFNγ, IL8, IL-12, IL-1ra, and MIP1α) were significantly higher in convalescent than in acute phase sera (Figure 1). This observation is similar to the previous studies showing elevated concentrations of proinflammatory cytokines/chemokines in the convalescent or asymptomatic human cases [12,13]. In contrast, the concentrations of the five cytokines/chemokines (e.g., IFNα, IP-10, MIP1β, IL-6, and TNFα) did not differ significantly between the two groups (Figure 2). Furthermore, the concentration of MCP-1, one of the proinflammatory chemokines, was lower in the convalescent than in the non-convalescent sera (Figure 2). These data indicated that IFN-γ, IL-8, IL-12, IL-1ra or MIP1α might therefore be involved with the host immune responses in the convalescent phase of RESTV infection.

Discussion

We previously developed a RESTV NP-specific IgG-ELISA and IFA that proved to be useful for the seroepidemiological studies of cynomolgus macaques during the RESTV epizootic in the Philippines in 1996 [6-8]. The assays based on recombinant NP are sensitive for the detection of RESTV-specific antibodies. On the other hand, anti-$GP_{1,2}$ antibodies are elicited in EBOV-infected human cases and are believed to have protective roles against lethal EBOV infection [21,22]. In the present study, in order to gain insight into the IgG responses during the recovery from infection with RESTV, anti-NP, anti-$GP_{1,2}$, and neutralizing antibodies and the level of viremia in the serum specimens were analyzed. The data presented herein showed that the anti-$GP_{1,2}$ response, rather than the anti-NP response, was correlated with both the lack in viremia and the neutralizing activities in the sera of RESTV-infected cynomolgus macaques. There may be at least two possibilities for the lack of anti-$GP_{1,2}$ IgG in the acute phase samples. It is known that soluble GP (sGP), which does not contain membrane anchor, is secreted during ebolaviruses infection, and it can absorb the anti-$GP_{1,2}$ antibodies [23]. The other possibility is that apoptosis of lymphocytes is induced during RESTV infection and the resulting host immune responses may thus be abrogated. Although the precise mechanism of action is still unknown, it is likely that no IgG responses to RESTV $GP_{1,2}$ are induced in the cynomolgus macaques during the acute phase of infection.

Since the cynomolgus macaques at the facility where the RESTV epizootic occurred were euthanized, sequential serum specimens from each cynomolgus macaque were not available. It is also difficult to determine when each cynomolgus macaque became infected with RESTV. We found that three specimens that have anti-GP antibodies were obtained from dead monkeys (#2180, 2181 and 2194), however, it is difficult to conclude whether RESTV infection caused their death because of the possibility of succumbing to infection by SHFV or some other agents. It is predicted that, among the serum samples examined here, nine were acute phase samples because they were positive in Ag-ELISA or immunohistochemistry [3,15] (Table 1). On the other hand, all but one (serum #2194) of the anti-$GP_{1,2}$-positive serum samples were Ag negative. Therefore, these cynomolgus macaques were considered to be in the convalescent phase of RESTV infection. In this regard, the presence of the anti-$GP_{1,2}$ antibody is thought to be a useful indicator for convalescence in cynomolgus macaques infected with RESTV.

Aberrant proinflammatory cytokines/chemokines are a significant factor implicated in the disease progression of EBOV- and SUDV-infected human cases and experimentally

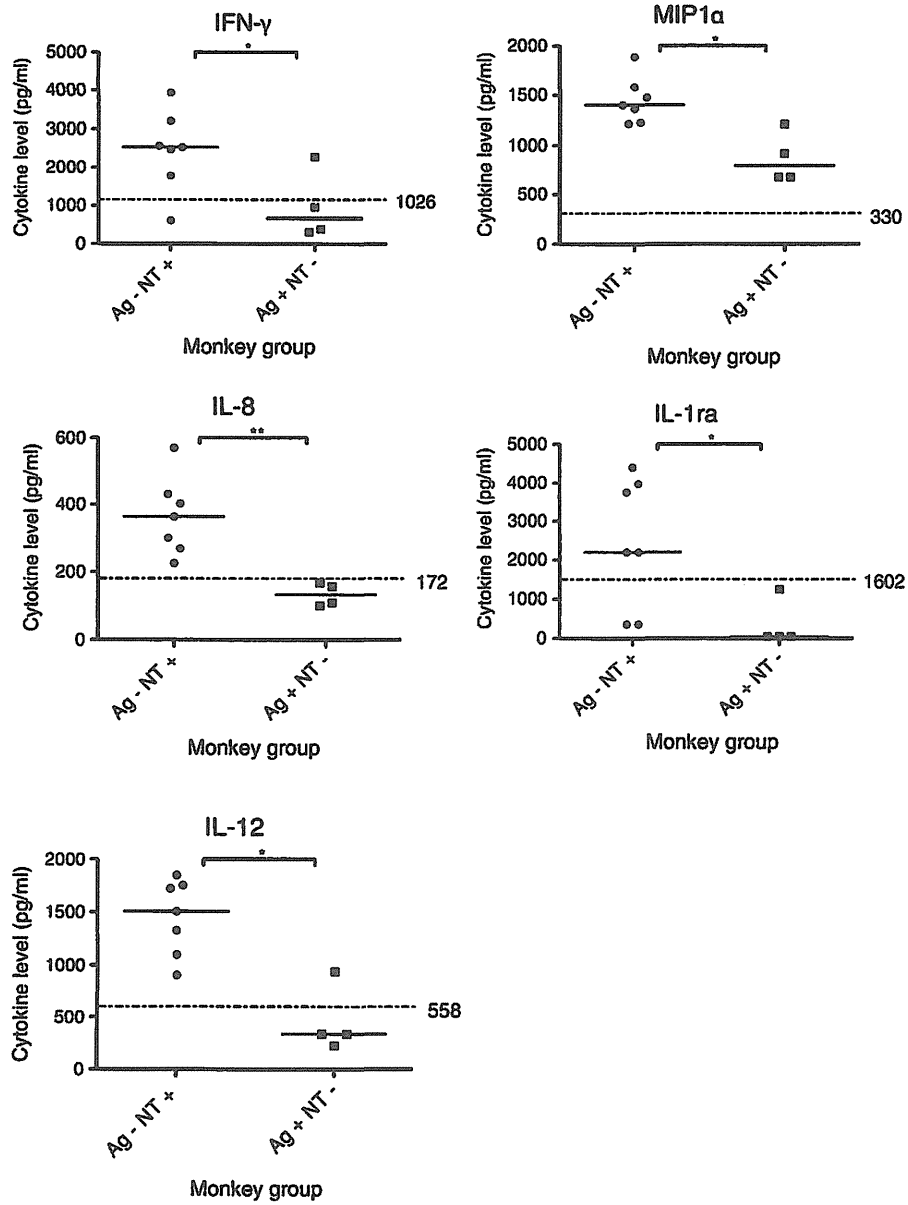

Figure 1 Profiles of the serum cytokine and chemokine concentrations in Reston virus-infected cynomolgus macaques. The serum concentrations of IFN-γ, IL-8, IL-12, MIP1α, and IL-1ra. The concentrations of these proinflammatory cytokines/chemokines (IFN-γ, IL-8, IL-12, MIP1α) and the anti-inflammatory cytokine (IL-1ra) were significantly higher in convalescent (Ag - NT +) than in non-convalescent sera (Ag + NT -). Each dot represents one sample, and dashes (-) represent the median values. * indicates $p < 0.05$, ** indicates $p < 0.005$ (Mann Whitney test). Broken lines and the numbers written aside indicate the average concentrations of cytokine and chemokine in negative control cynomolgus macaques (n = 13).

infected cynomolgus macaques [10,11,24]. In addition, a balanced proinflammatory response is believed to be a critical factor for determining the disease outcome [25,26]. We focused on the circulating inflammatory cytokines/chemokines in RESTV-infected cynomolgus macaques and examined their relationship with convalescence. We thus found the concentrations of several proinflammatory cytokines/chemokines, such as IFNγ, IL8, IL-12, and MIP1α, to be significantly higher in convalescent sera than in non-convalescent sera. Gupta et al. (2012) recently demonstrated that convalescent serum samples obtained from BDBV-infected human cases include high concentrations of IL-1α, IL1β, IL6, TNFα, and MCP-1 [13]. Although the exact profiles of proinflammatory cytokines/chemokines shown in our study are different from those reported by Gupta et al., these differences are considered most likely to be due to

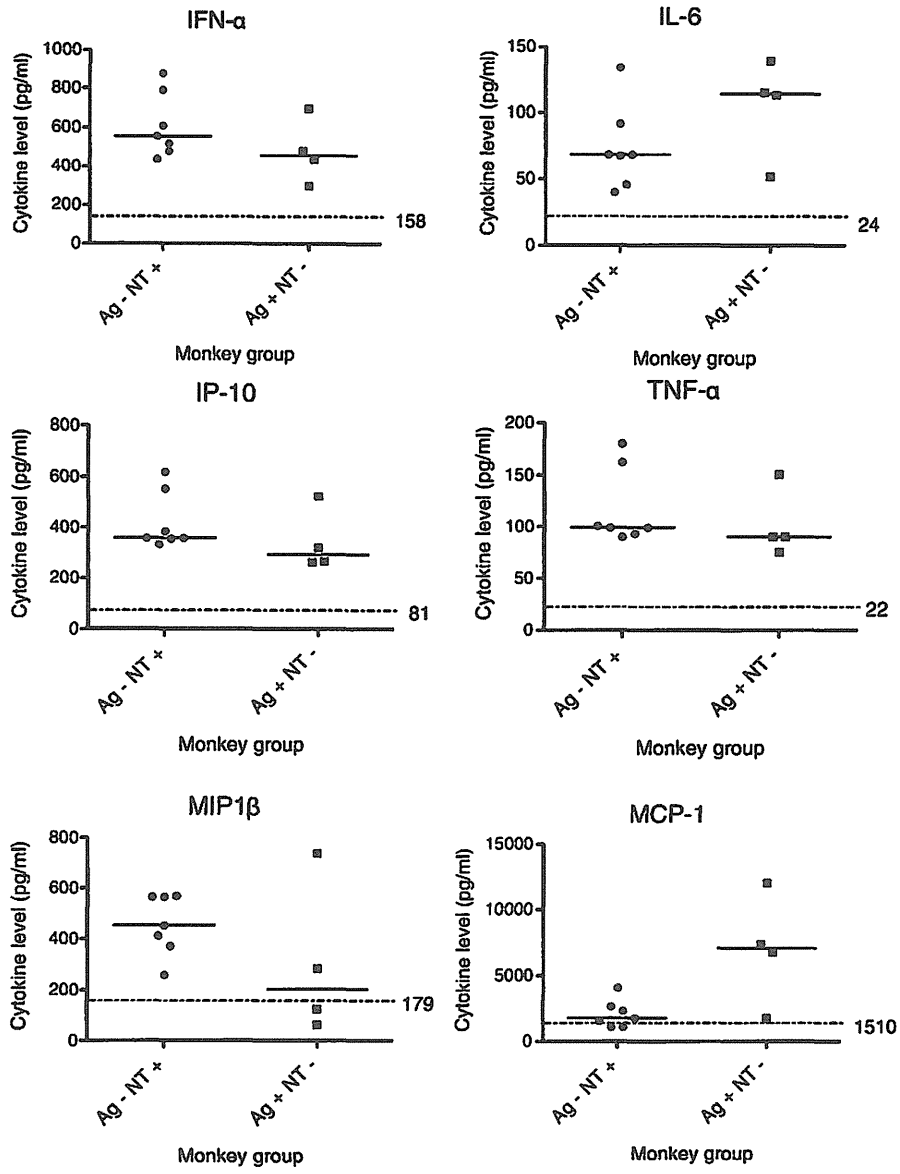

Figure 2 Profiles of the serum cytokine and chemokine concentrations in Reston virus-infected cynomolgus macaques. The IFN-α, IP-10, TNF-α, MIP1β, IL-6 and MCP-1 concentrations. The serum concentrations of these cytokines/chemokines (IFN-α, IP-10, TNF-α, MIP1β) did not differ significantly between the convalescent cases (Ag - NT +) and the non-convalescent cases (Ag + NT -). The concentration of the anti-inflammatory cytokine, MCP-1, was lower in convalescent than in non-convalescent cynomolgus macaques (Mann Whitney test). Each dot represents one sample, and dashes (-) represent the median values. Broken lines and the numbers written aside indicate the average concentrations of cytokine and chemokine in negative control cynomolgus macaques (n = 13).

differences among ebolaviruses (RESTV vs. BDBV), host species (cynomolgus macaques vs. humans), and differences related to the disease phase when the samples were obtained. It is possible that the upregulation of the proinflammatory innate immune responses contributed to the recovery from RESTV infection in cynomolgus macaques.

In rhesus monkeys experimentally infected with a lethal dose of EBOV, anti-inflammatory cytokines, such as IL-13 and IL-1ra, are highly elevated in the acute phase [11]. In human Ebola VHF patients, increased concentrations of IL-10 and IL-1ra have been shown in fatal cases, thus suggesting that the mixed anti-inflammatory response syndrome (MARS) contributes to the pathogenesis of the hemorrhagic fever caused by ebolaviruses. Since all of the cynomolgus macaques involved in the epizootic were euthanized at the affected facility, the actual fate of the cynomolgus macaques was not clear, and some might have survived the infection. Our data obtained using the sera

from cynomolgus macaques in the RESTV epizootic showed higher IL-1ra responses in the convalescent phase than in the non-convalescent phase (Figure 1). There were no significant differences in the concentration of IL-10 between the two groups (data not shown). This suggests that, unlike other ebolaviruses infections, RESTV does not induce MARS, which is characterized by an elevated induction of IL1ra in the acute phase.

In conclusion, we have shown that the anti-$GP_{1,2}$ responses, rather than the anti-NP responses, in cynomolgus macaques naturally infected with RESTV were specifically detected in the convalescent stage of RESTV infection. In addition, a high concentration of proinflammatory cytokines/chemokines was observed in the convalescent phase. Therefore, the anti-$GP_{1,2}$ response and the upregulation of the specific proinflammatory response might be useful indicators of convalescence from RESTV infection in cynomolgus macaques.

Conclusions

In this study, we analyzed the humoral responses in cynomolgus macaque serum samples collected during the 1996 Reston outbreak in the Philippines and demonstrated that the anti-RESTV $GP_{1,2}$ response and the proinflammatory innate response play significant roles in the convalescence from RESTV infection in cynomolgus macaques.

Methods

Sera

Twenty-seven cynomolgus macaque serum samples were obtained from the cynomolgus macaque facility in the Philippines where the 1996 RESTV epizootic occurred [27]. The serum specimens in the affected facility were collected under quarantine of the Philippines. Nineteen of the 27 samples were previously subjected to an antigen capture ELISA. Nine of the samples were found to be RESTV antigen-positive, and the remaining 10 were considered to be antigen-negative [15]. The serum specimens were treated at 56°C for 30 minutes and virus in the cynomolgus macaque serum samples were inactivated. As negative controls for the IgG-ELISA and IFA, we used serum samples from 102 cynomolgus macaques collected at the Tsukuba Primate Research Center (TPRC) in Japan. As positive controls for the IgG-ELISA and IFA, two rabbits were immunized four times with the histidine-tagged ectodomain of the RESTV glycoprotein ($GP_{1,2}$) (RESTV $GP_{1,2}\Delta TM$). The histidine-tagged RESTV $GP_{1,2}\Delta TM$ of a 1996 RESTV [28] was prepared and purified as described below. The sera were collected from the rabbits, inactivated, and stored at 4°C until use. The experiments with animals were performed in accordance with the Animal Experimentation Guidelines of the National Institute of Infectious Diseases. The protocol was approved by the Institutional Animal Care and Use Committee of the institute (Permit number: 990163 and 109075).

Expression and purification of the recombinant RESTV $GP_{1,2}$ ectodomain

A recombinant baculovirus that expresses RESTV $GP_{1,2}\Delta TM$ was used to prepare recombinant RESTV $GP_{1,2}$ for the IgG-ELISA [16]. Briefly, a recombinant baculovirus carrying the ectodomain of RESTV $GP_{1,2}$ (DDBJ accession no. AB050936) with histidine-tag sequences at its 3'-terminus was infected into Tn5 cells at a multiplicity of infection (moi) of 1. The cells were collected, washed with PBS, and then lysed in PBS containing 1% Nonidet P40 (NP-40) on ice for 15 min. After being centrifuged, the recombinant RESTV $GP_{1,2}\Delta TM$ was purified with Ni^{2+}-agarose beads (QIAGEN, Hilden, Germany) and His Bind Kits (Novagen, Darmstadt, Germany). The purified recombinant RESTV $GP_{1,2}\Delta TM$ was used for the IgG-ELISA specific for RESTV $GP_{1,2}$. Lysates of Tn5 cells infected with baculovirus with a deleted polyhedrin gene, Ac-ΔP, were similarly processed and then used as negative control antigen in the IgG-ELISA described below.

RESTV $GP_{1,2}$-specific IgG-ELISA

Ninety-six well plates were coated with the RESTV $GP_{1,2}\Delta TM$ or with negative control antigen in 100 μl of PBS and incubated overnight at 4°C. The plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T), and then 200 μl of PBS-T containing 5% skim milk (SKIM-PBS-T) was added to each well and incubated for 2 hr at 37°C. The cynomolgus macaque sera were diluted at 1:100, 1:400, 1:1,600, and 1:6,400 in SKIM-PBS-T, and the hyperimmune rabbit sera were four-fold serially diluted from 1:1,000 to 1:64,000 in SKIM-PBS-T. One hundred microliters of each serum dilution was added to the antigen-coated wells and incubated for 1 hr at 37°C. After they were washed three times with PBS-T, the wells were further reacted with either HRP-conjugated goat anti-human IgG (H + L) (Lot:60504974, ZyMED) or HRP-conjugated goat anti-rabbit IgG (H + L) (Lot:398581A, ZyMED) at a dilution of 1:1,000 in SKIM-PBS-T. After being washed three times again with PBS-T, the ABTS substrate (Roche Diagnostics) was added to the wells. Then, the plates were incubated for 30 minutes at 37°C, and the OD values of the wells at 405 nm were measured. Adjusted OD values were calculated by subtracting the OD value of the wells coated with the negative control antigen from that of the wells coated with RESTV $GP_{1,2}\Delta TM$.

RESTV NP-specific IgG-ELISA

The NP-specific IgG-ELISA, which is similar to the $GP_{1,2}$-specific ELISA except for the purified recombinant

RESTV NP with a histidine tag at the C-terminus, has been previously reported [6,16].

ELISA index and determination of the cut-off value for the IgG-ELISA

The sum of the OD values of serum dilutions at 1:100, 1:400, 1:1,600, and 1:6,400 for each specimen was calculated and designated as an "ELISA index" in the IgG-ELISA. The mean plus three standard deviations (SD) of the ELISA indices for the IgG-ELISAs was calculated using serum samples from uninfected TPRC cynomolgus macaques and was used as the cut-off value for the IgG-ELISAs.

Indirect immunofluorescent antibody assay (IFA) specific for RESTV NP and $GP_{1,2}$

The IFA specific for RESTV NP was reported previously [8]. In the present study, a RESTV $GP_{1,2}$-specific IFA was established using stably RESTV $GP_{1,2}$-expressing HeLa cells. HeLa cell line was purchased from the American Type Culture Collection and used. The RESTV $GP_{1,2}$ cDNA of a 1996 RESTV was subcloned into a mammalian expression plasmid, pKS336, to generate pKS336-RESTV-$GP_{1,2}$. The HeLa cells expressing RESTV $GP_{1,2}$ were selected in a medium containing 2 μg/ml of blasticidin-S-hydrochloride (Sigma, St. Lois, MO) after transfection with pKS336-RESTV-$GP_{1,2}$ using the FuGENE HD Transfection Reagent (Roche Diagnostics, Germany). The cells were trypsinized, washed with PBS, and mixed with normal HeLa cells, and were then spotted on 14 well Teflon-coated glass slides, air dried, and fixed with acetone at room temperature for 5 min. The slides were stored at -80°C until use.

The slides were thawed and dried just before use. The serum specimens were 2-fold serially diluted in PBS, and a 20 μl aliquot of each dilution was applied to the wells of the antigen slides and incubated at 37°C for 1 hr in a humidified chamber. Then the antigen slides were washed with PBS and reacted with 20 μl per well of FITC-conjugated goat anti-human IgG (H + L) (ZyMax lot: 415460A, Invitrogen, CA, U.S.A.) for cynomolgus macaque sera and FITC-conjugated goat anti-rabbit IgG (H + L) (ZyMax lot: 402686A, Invitrogen, CA, U.S.A.) for rabbit hyperimmune sera at a dilution of 1:100. After incubation at 37°C for 1 hr, the slides were washed with PBS and covered with micro cover glasses. The slides were examined for the staining pattern under a fluorescent microscope. The antibody titer in the IFA was determined as the reciprocal of the highest dilution showing positive staining.

RESTV neutralization (NT) assay using VSV-RESTV-$GP_{1,2}$/GFP

The VSV pseudotype bearing RESTV $GP_{1,2}$, VSV-RESTV-$GP_{1,2}$/GFP was generated essentially according to the method described for the VSV pseudotype bearing SARS-CoV S protein [29], except that pKS336-RESTV-$GP_{1,2}$ was used in the present study [17]. Briefly, 293 T cells were prepared in 24 well plates at 20-30% confluency. The cells were transfected with pKS336-RESTV-$GP_{1,2}$ using FuGENE HD. The cells were then cultured for 24 hr and inoculated with VSV ΔG*/GFP pseudotyped with the VSV-G protein at a moi of 5, adsorbed for 1 hr at 37°C, and then washed with DMEM-5% FCS and cultured for 24 hr. The culture supernatants were collected and centrifuged at 1,000 rpm to remove cell debris. Thereafter, the supernatants were stored at -80°C as VSV-RESTV-$GP_{1,2}$/GFP. The infectivity titer of VSV-RESTV-$GP_{1,2}$/GFP, harboring the VSV ΔG*/GFP genome, was determined by counting the number of GFP-positive cells under a fluorescent microscope upon infection into Vero E6 cells, as described previously. Briefly, VSV-RESTV-$GP_{1,2}$/GFP was 3.2 (0.5 $\log_{10}$)-fold serially diluted with DMEM-5% FCS and then inoculated to Vero E6 cells seeded in 96 well culture plates. The cells were incubated at 37°C in a CO_2 incubator for 24 hr. Then, GFP-positive cells were detected and counted under a fluorescent microscope (BZ-9000; KEYENCE, Osaka, Japan), and the infectious units (IU) of the pseudotyped VSV were calculated.

The serum samples were serially diluted in DMEM-5% FCS, and a 50 μl aliquot of each dilution was mixed with the same volume of DMEM-5% FCS containing 1,000 IU of VSV-RESTV-$GP_{1,2}$/GFP and incubated for 1 hr at 37°C. The mixture was inoculated into Vero E6 cells and incubated for 24 hr. The number of GFP-positive infected cells was counted, and serum dilutions with 50% neutralization (NT_{50}) were identified.

Multiplex assay for cytokines and chemokines in the cynomolgus macaque sera

Eleven RESTV-infected cynomolgus macaque serum samples were inactivated at 56°C for 30 min, diluted 1:10 in the assay diluent supplied with the Human Cytokine 25-Plex antibody bead kit (Invitrogen, CA), and were subjected to a multiplex cytokine analysis using a Luminex 100 instrument (Luminex Co., Austin, TX) according to the manufacturer's instructions. This Human Cytokine 25-Plex antibody bead kit was previously used to cynomolgus macaque sera and the cross-reactivity was confirmed [30]. As negative controls, we used sera from 13 cynomolgus macaques bred at the TPRC and investigated the cytokine concentrations of these serum samples.

Abbreviations

Ag: Antigen; BDBV: Bundibugyo ebolavirus; EBOV: Ebola virus; ELISA: Enzyme-linked immunosorbent assay; $GP_{1,2}$: Glycoprotein; $GP_{1,2}$ΔTM: Ectodomain of the RESTV glycoprotein; VHF: Viral hemorrhagic fever;
IFA: Immunofluorescent antibody assay; MARS: Mixed anti-inflammatory response syndrome; NP: Nucleoprotein; NP-40: Nonidet P40;
NT: Neutralization; PBS-T: PBS containing 0.05% Tween 20; RESTV: Reston

virus; SD: Standard deviation; SHFV: Simian hemorrhagic fever virus; SKIM-PBS-T: PBS-T containing 5% skim milk; TPRC: Tsukuba Primate Research Center; VSV: Vesicular stomatitis Indiana virus; VSV-RESTV-$GP_{1,2}$/GFP: VSV pseudotype bearing RESTV $GP_{1,2}$.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
ST, TI, SF, and SM designed the experiments and analyzed the experimental data. ST and SF prepared the manuscript. SM supervised the experiments and helped draft the manuscript. YS, SW, and II helped to perform the experiments. NN performed the multiplex assay. YY and MM prepared the serum samples. TI, TM, YI, MS, HA and SK supervised the experiments. All authors have read and approved the final manuscript.

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BRIEF REPORT

Genomic and serological detection of bat coronavirus from bats in the Philippines

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Abstract Bat coronavirus (BtCoV) is assumed to be a progenitor of severe acute respiratory syndrome (SARS)-related coronaviruses. To explore the distribution of BtCoVs in the Philippines, we collected 179 bats and detected viral RNA from intestinal or fecal samples by RT-PCR. The overall prevalence of BtCoVs among bats was 29.6 %. Phylogenetic analysis of the partial RNA-dependent RNA polymerase gene suggested that one of the detected BtCoVs was a novel alphacoronavirus, while the others belonged to the genus *Betacoronavirus*. Western blotting revealed that 66.5 % of bat sera had antibodies to BtCoV. These surveys suggested the endemic presence of BtCoVs in the Philippines.

Keywords Bat coronavirus · Coronavirus · Bats · Antibody · Viruses · SARS · Severe acute respiratory syndrome · Zoonosis

Abbreviations

BtCoV	Bat coronavirus
CoV	Coronavirus
SARS	Severe acute respiratory syndrome
RT-PCR	Reverse transcriptase polymerase chain reaction
RdRp	RNA–dependent RNA polymerase
nt	Nucleotide
N	Nucleocapsid
PBS	Phosphate-buffered saline
WB	Western blotting
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
CBB	Coomassie brilliant blue
HRP	Horseradish peroxidase

Recently, bats have been recognized as a major reservoir of emerging viral infections that cause serious diseases in humans and other mammals [1]. Several viruses that cause severe diseases in humans have been detected in bats, such as lyssaviruses related to rabies virus, Hendra virus, Nipah virus, Ebola virus, Marburg virus and severe acute respiratory syndrome (SARS) coronavirus [1]. Hence, there is a growing focus on bats as zoonotic hosts.

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SARS was caused by a previously unknown coronavirus [2]. From 2002 to 2003, the SARS epidemic affected mainly China, Hong Kong and Taiwan, afflicting about 8000 people and killing over 600 [3]. Investigations of bats revealed that horseshoe bats (*Rhinolophus*) in southern China were natural reservoirs of a coronavirus related to SARS-CoV [4, 5].

This prompted surveys of bat coronaviruses (BtCoVs) worldwide, and novel viruses were detected in North and South America, Africa, Europe and Asia [6–12], revealing a broad diversity of BtCoVs. Molecular phylogenetic analysis suggested that all mammalian coronaviruses including SARS-CoV were likely to have evolved from BtCoVs [13].

We previously reported that two groups of BtCoVs were detected in the Philippines: one belonging to the genus *Alphacoronavirus* and the other belonging to the genus *Betacoronavirus* [14]. To explore the diversity of BtCoVs in the Philippines, we carried out more surveys in this study. We investigated 179 intestinal or fecal samples to detect coronaviral RNA and performed phylogenetic analysis on isolated coronavirus sequences. We also detected virus-specific antibodies, indicating how widely distributed BtCoVs are in the Philippines.

Table 1 Prevalence of coronavirus in bat fecal samples detected by RT-PCR and WB analysis

Year	Location	Species	RT-PCR positive	WB positive	Samples tested
2009#	Site A	Lesser dog-faced fruit bat (*Cynopterus brachyotis*)	1	1	2
		Greater musky fruit bat (*Ptenochirus jagori*)	5	10	10
		Philippine sheath-tailed bat (*Emballonura alecto*)	2	5	8
		Large rufous horseshoe bat (*Rhinolophus rufus*)	1	2	2
		Philippine pygmy fruit bat (*Haplonicteris fischeri*)	0	1	6
		Lesser long-tongued fruit bat (*Macroglossus minimus*)	0	1	2
		Total	9	20	30
	Site B	Lesser dog-faced fruit bat (*Cynopterus brachyotis*)	1	4	6
		Greater musky fruit bat (*Ptenochirus jagori*)	1	6	8
		Geoffroy rousette bat (*Rousettus amplexicaudatus*)	1	4	5
		Total	3	14	19
2010*	Site C	Lesser dog-faced fruit bat (*Cynopterus brachyotis*)	16	32	43
		Greater musky fruit bat (*Ptenochirus jagori*)	1	6	11
		Geoffroy rousette bat (*Rousettus amplexicaudatus*)	0	0	1
		Diadem roundleaf bat (*Hipposideros diadema*)	0	0	3
		Total	17	38	58
	Site D	Lesser dog-faced fruit bat (*Cynopterus brachyotis*)	11	17	28
		Greater musky fruit bat (*Ptenochirus jagori*)	4	14	21
		Diadem roundleaf bat (*Hipposideros diadema*)	1	0	1
		Total	16	31	50
2011*	Site E	Lesser dog-faced fruit bat (*Cynopterus brachyotis*)	2	4	4
		Greater musky fruit bat (*Ptenochirus jagori*)	5	12	17
		Lesser long-tongued fruit bat (*Macroglossus minimus*)	1	0	1
		Total	8	16	22
Total		Lesser dog-faced fruit bat (*Cynopterus brachyotis*)	31	58	83
		Greater musky fruit bat (*Ptenochirus jagori*)	16	48	67
		Philippine sheath-tailed bat (*Emballonura alecto*)	2	5	8
		Large rufous horseshoe bat (*Rhinolophus rufus*)	1	2	2
		Philippine pygmy fruit bat (*Haplonicteris fischeri*)	0	1	6
		Lesser long-tongued fruit bat (*Macroglossus minimus*)	1	1	3
		Geoffroy rousette bat (*Rousettus amplexicaudatus*)	1	4	6
		Diadem roundleaf bat (*Hipposideros diadema*)	1	0	4
		Total	53	119	179

intesteinal and * fecal samples were examined by RT-PCR

A total of 179 bats (8 species) were captured at five different sites in the Philippines during the months of March 2009, August 2010 and May 2011. Each sampling was performed after receiving permission from the Government of the Philippines. For anesthesia, 15 mg/kg tiletamine and zolazepam (Virbac, Carros, France) were injected intraperitoneally into captured bats, and the anesthetized bats were sacrificed by cardiac exsanguination. Liver, lienal, renal, lung, fecal and intestinal specimens were collected from each captured bat and were shared with the coworkers. All of the experiments using animals were conducted under the supervision of, and with approval from the Animal Care and Use Committee of the Faculty of Agriculture, the University of Tokyo.

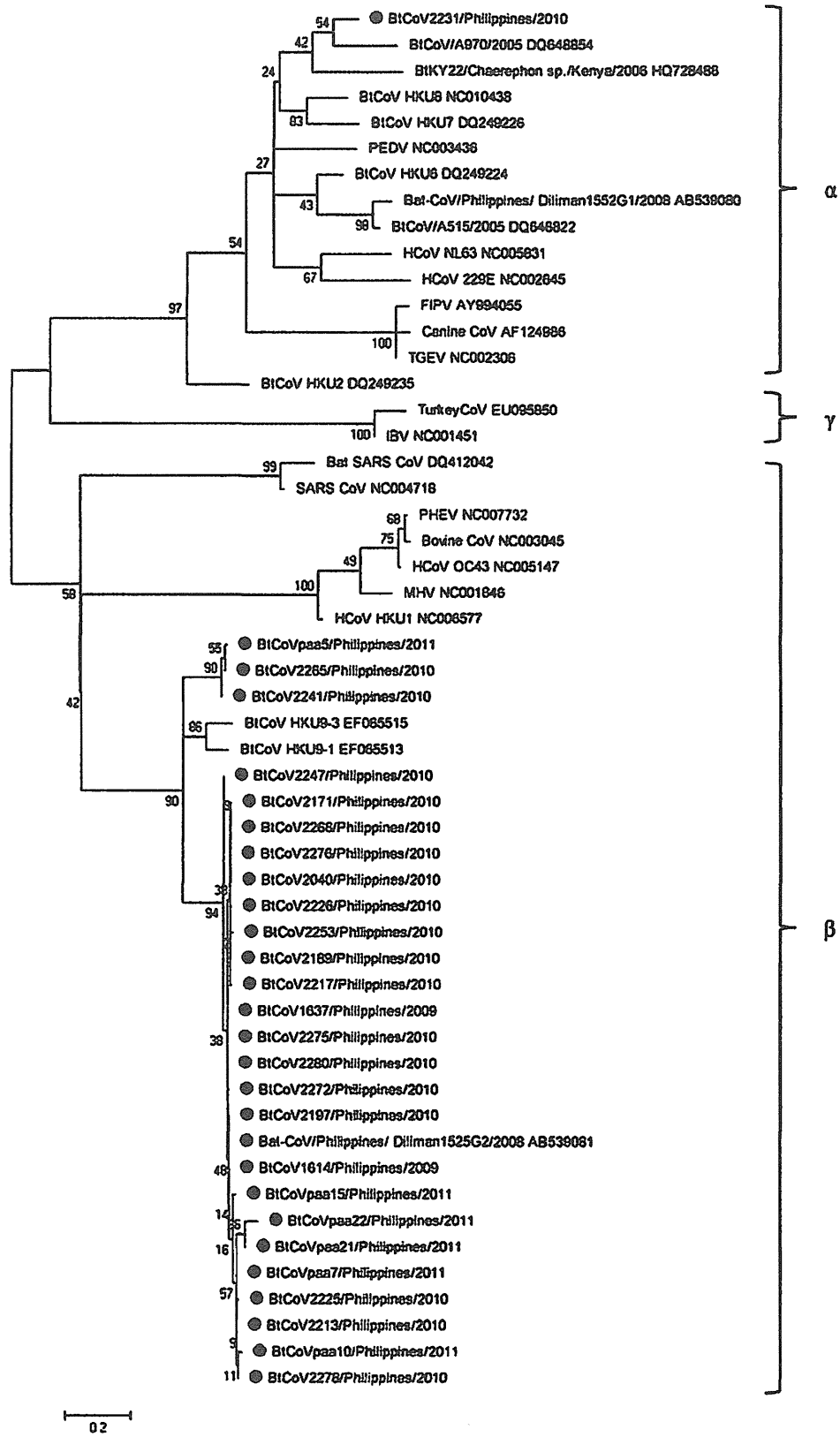
To determine the presence of viral RNA in bat samples, reverse transcriptase polymerase chain reaction (RT-PCR) was performed with a pair of consensus primers targeted to a highly conserved region of the RNA-dependent RNA polymerase (*RdRp*) gene [10]. Viral RNA was extracted from feces or intestinal tissue of the bats using a Total RNA Isolation Mini Kit (Agilent Technologies, Santa Clara, CA, USA). Complementary DNA was synthesized using a SuperScript III Kit (Invitrogen, Carlsbad, CA, USA). PCR was performed using GoTaq PCR Master Mix (Promega, Madison, WI, USA) and 0.2 μM primers (primer 1, 5′-GGTTGGGACTATCCTAAGTGTGA-3′; primer 2, 5′-CCATCATCAGATAGAATCATCATA-3′). PCR products were analyzed by agarose gel electrophoresis. Amplicons (440 nucleotides [nt] long) were gel–purified and directly sequenced with an ABI 3130 XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

To detect anti-BtCoV antibodies in bat sera, western blot (WB) analysis was performed. Recombinant BtCoV nucleocapsid (N) protein produced by prokaryotic expression was used as an antigen. To amplify the full-length BtCoV N protein–coding region (1389 nt; AB543561), PCR was performed using KOD–Plus (Toyobo, Osaka, Japan) and 0.3 μM primers (forward primer, 5'.GAACAGATTGG AGGTATGTCTGGACGGAATAAGC-3'; reverse primer, 5'-TGCTCGAGTGCGGCCTTAGGATCTCTCATTAGC A-3'). The amplified fragment was cloned into the pE-SUMOstar vector (LifeSensors, Malvern, PA, USA). *E. coli* strain BL21 (DE3) was transformed with the recombinant plasmid. N protein expression was induced by the addition of 1 mM isopropyl–β–D–thiogalactopyranoside. The purity of proteins was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Coomassie brilliant blue (CBB) staining and WB analysis.

Recombinant BtCoV N protein was separated by 8 % SDS-PAGE. After blotting, nitrocellulose membranes were cut into strips and incubated for 1 h with bat serum samples (diluted 1:1000) or horseradish peroxidase (HRP)-conjugated anti-6x His tag antibody (Abcam, Cambridge, UK) at a dilution of 1:5000. The strips were rinsed and further incubated for 1 h with rabbit anti-bat IgG antibody (diluted 1:1000) [15] and subsequently incubated for 1 h with HRP-conjugated donkey anti-rabbit IgG (diluted 1:2000; GE Healthcare, Milwaukee, WI, USA). Signals were detected using an Enhanced Chemiluminescence (ECL) Detection Kit (GE Healthcare).

From 2009 to 2011, we sampled a total of 179 bats at five sites in the Philippines. To detect viral RNA, RT-PCR was performed using a pair of primers for a conserved 440-nt region within the coronavirus *RdRp* gene. Intestinal samples containing feces obtained in 2009 and feces collected in 2010 and 2011 were examined. As shown in Table 1, the overall prevalence of BtCoV was 53/179 (29.6 %). Amplified fragments were sequenced directly for characterization. A BLAST search (http://blast.ncbi.nlm.nih.gov/Blast) suggested that most of the sequences obtained in this study were similar to the sequence we reported previously (BtCoV/Philippines/Diliman1525G2/2008). These sequences were >98 % similar to one another and were obtained from lesser dog-faced fruit bats (*Cynopterus brachyotis*) and greater musky fruit bats (*Pteropochirus jagori*). Sequences from greater musky fruit bats (5/16 specimens positive by RT-PCR) that were similar to BtCoV/Philippines/Diliman1525G2/2008 were also found (87 % identical at the nucleotide level and 95 % identical in their translated amino acid sequences). Sequences that were >98 % identical were also amplified from Philippine sheath-tailed bats (*Emballonura alecto*), large rufous horseshoe bats (*Rhinolophus rufus*), Geoffroy's rousette bats (*Rousettus amplexicaudatus*), and lesser long-tongued fruit bats (*Macroglossus minimus*). A novel sequence was detected in a diadem roundleaf bat (*Hipposideros diadema*), which had 82 % similarity to BtCoV/A977/2005, found in China [16], and 75 % to the BtCoV we reported previously (BtCoV/Philippines/Diliman1552G1/2008) [14]. Phylogenetic reconstruction from isolated sequences was performed using MEGA5 software [17] (Fig. 1). This suggested that one of the detected sequences (from the diadem roundleaf bat) was related to members of the genus *Alphacoronavirus* (BtCoV2231/Philippines/2010), while most of the BtCoVs belonged to the genus *Betacoronavirus* (BtCoV/Philippines/Diliman1525G2/2008 and BtCoV2265/Philippines/2010). The two sequences (BtCoV2265/Philippines/2010 and BtCoV2231/Philippines/2010) were deposited into the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/) with the accession numbers AB683970 and AB683971, respectively.

WB analysis was performed to detect antibodies to the CoV N protein. The 6x His-SUMO–fused full–length N protein was expressed in BL21 (DE3) cells and used as antigen. The expression of the ~63-kDa recombinant


BtCoV2231/Philippines/2010
BtCoV/A970/2005 DQ648854
BtKY22/Chaerephon sp./Kenya/2006 HQ728488
BtCoV HKU8 NC010438
BtCoV HKU7 DQ249226
PEDV NC003436
BtCoV HKU6 DQ249224
Bat-CoV/Philippines/ Diliman1552G1/2008 AB539080
BtCoV/A515/2005 DQ648822
HCoV NL63 NC005831
HCoV 229E NC002645
FIPV AY994055
Canine CoV AF124986
TGEV NC002306
BtCoV HKU2 DQ249235
TurkeyCoV EU095850
IBV NC001451
Bat SARS CoV DQ412042
SARS CoV NC004718
PHEV NC007732
Bovine CoV NC003045
HCoV OC43 NC005147
MHV NC001846
HCoV HKU1 NC006577
BtCoVpaa5/Philippines/2011
BtCoV2265/Philippines/2010
BtCoV2241/Philippines/2010
BtCoV HKU9-3 EF065515
BtCoV HKU9-1 EF065513
BtCoV2247/Philippines/2010
BtCoV2171/Philippines/2010
BtCoV2268/Philippines/2010
BtCoV2276/Philippines/2010
BtCoV2040/Philippines/2010
BtCoV2226/Philippines/2010
BtCoV2253/Philippines/2010
BtCoV2189/Philippines/2010
BtCoV2217/Philippines/2010
BtCoV1637/Philippines/2009
BtCoV2275/Philippines/2010
BtCoV2280/Philippines/2010
BtCoV2272/Philippines/2010
BtCoV2197/Philippines/2010
Bat-CoV/Philippines/ Diliman1525G2/2008 AB539081
BtCoV1614/Philippines/2009
BtCoVpaa15/Philippines/2011
BtCoVpaa22/Philippines/2011
BtCoVpaa21/Philippines/2011
BtCoVpaa7/Philippines/2011
BtCoV2225/Philippines/2010
BtCoV2213/Philippines/2010
BtCoVpaa10/Philippines/2011
BtCoV2278/Philippines/2010
α
γ
β
0.2