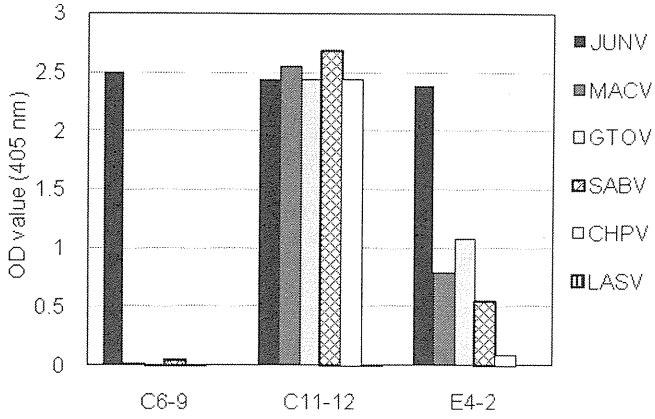

FIG. 1. Reactivity of each MAb with arenavirus rNP. Each purified rNP (100 ng/well) was used to coat microplates as described in the text, and the reactivities of each MAb to the rNPs of JUNV, MACV, GTOV, SABV, CHPV, and LASV were measured. The MAbs are shown on the *x* axis. Results are expressed as the OD_{405}.

Reactivities of MAbs to rNPs of arenaviruses. The reactivities of MAbs to the rNPs of human pathogenic arenaviruses were examined by ELISA. MAb C6-9 reacted specifically with the rNP of JUNV but did not react with those of the other pathogenic South American arenaviruses (Fig. 1). On the other hand, MAb C11-12 reacted at the same level with the rNPs of all of the pathogenic South American arenaviruses, including JUNV, GTOV, MACV, SABV, and CHPV. MAb E4-2 reacted strongly with the rNP of JUNV, slightly more weakly with those of GTOV, MACV, and SABV, and very weakly with that of CHPV. However, MAb E4-2 reacted clearly with the rNP of CHPV when ELISA plate wells were coated with more-concentrated CHPV Ag (data not shown). None of the three MAbs reacted with the rNP of the human pathogenic Old World arenavirus LASV.

Reactivity was also examined by IFA. Consistent with the ELISA result, MAb C6-9 reacted only with HeLa cells expressing the rNP of JUNV and MAb C11-12 reacted with HeLa cells expressing the rNPs of all of the pathogenic South American arenaviruses (Table 1). On the other hand, MAb E4-2, which showed cross-reactivity to other arenaviruses by ELISA, reacted only with HeLa cells expressing the rNP of JUNV (Table 1). None of the three MAbs reacted with LASV NP-expressing HeLa cells (Table 1).

Development of Ag capture ELISAs. Ag capture ELISAs were developed by using three MAbs as capture antibodies, and sensitivity and specificity were determined. The Ag capture ELISA with MAb C6-9 specifically detected the rNP of JUNV, whereas it could not detect the rNPs of the other South American arenaviruses. No less than 62.5 ng/well of the rNP of JUNV was detected by the Ag capture ELISA using MAb C6-9 (Fig. 2A). On the other hand, the Ag capture ELISAs using

TABLE 1. MAb reactivity with NPs of arenaviruses in IFA

MAb	Reactivity[a] with NP of:					
	JUNV	MACV	GTOV	SABV	CHPV	LASV
C6-9	+	−	−	−	−	−
C11-12	+	+	+	+	+	−
E4-2	+	−	−	−	−	−

[a] The symbols + and − indicate positive and negative reactions, respectively. The expression of each NP in HeLa cells was confirmed by IFA with a rabbit polyclonal antibody produced against each NP.

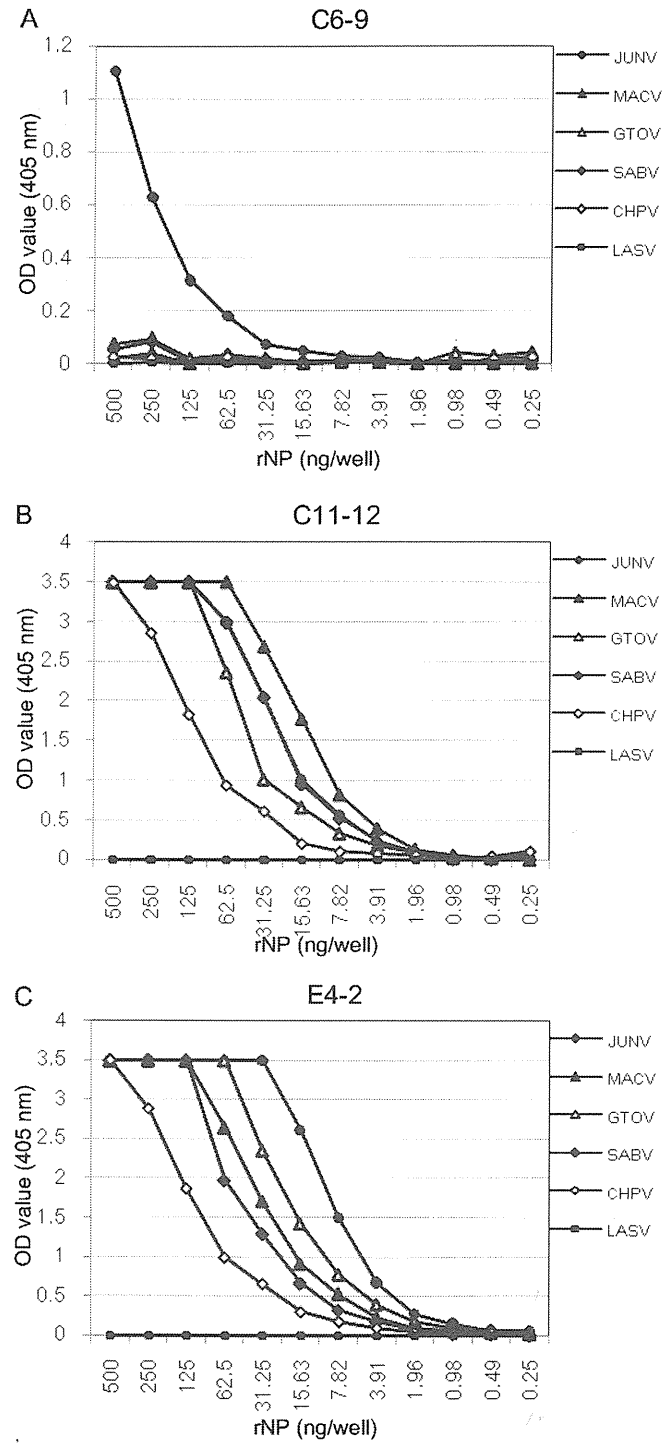

FIG. 2. Reactivity of each MAb in an Ag capture ELISA. Purified MAbs C6-9 (A), C11-12 (B), and E4-2 (C) were used to coat microplates as described in the text, and their abilities to capture the rNPs of JUNV, MACV, GTOV, SABV, CHPV, and LASV were examined at various concentrations in the Ag capture format. Results are expressed as the OD_{405}.

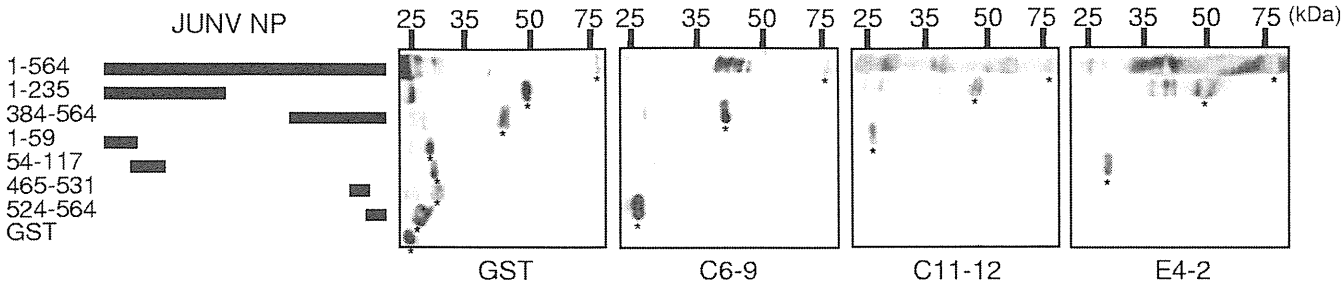

FIG. 3. Reactivities of MAbs C6-9, C11-12, and E4-2 with the GST-tagged JUNV NP by Western blotting. Schematic drawings of polypeptides of the JUNV NP and the amino acid positions of the polypeptides are shown on the left. The reactivities of MAbs and a control MAb against GST to these polypeptides by Western blotting are shown on the right. The asterisks indicate the polypeptides to which the MAbs reacted.

MAbs C11-12 and E4-2 were more sensitive at detecting the rNP of JUNV, with detection limits of 7.82 and 3.91 ng/well, respectively, and these Ag capture ELISAs also detected the rNPs of the other South American arenaviruses GTOV, MACV, SABV, and CHPV (Fig. 2B and C). In contrast, the LASV NP was not detected by any of the Ag capture ELISAs.

Determination of the epitope on the JUNV rNP recognized by the MAbs. In order to determine regions including epitopes on the JUNV rNP recognized by the MAbs, the reactivity of these MAbs was tested by Western blotting using the GST-JUNV frNP and a series of GST-JUNV trNPs as Ags. MAb C6-9 reacted with GST-JUNV frNP and trNPs at aa 384 to 564 and 524 to 564 (Fig. 3). MAb C11-12 reacted with GST-JUNV frNP and trNPs at aa 1 to 235 and 1 to 59 (Fig. 3). MAb E4-2 reacted with GST-JUNV frNP and trNPs at aa 1 to 235 and 54 to 117 (Fig. 3).

To further determine exact epitope positions on the rNP of JUNV, we performed epitope-blocking ELISAs with a series of overlapping synthetic peptides. As shown in Fig. 4A, peptides containing PPSLLFLP (aa 551 to 558) blocked the reaction of MAb C6-9 with the purified rNP of JUNV. Similarly, peptides containing WTQSLR (aa 12 to 17) blocked the reaction of MAb C11-12 with the purified rNP of JUNV (Fig. 4B).

Because the epitope recognized by MAb E4-2 could not be determined by epitope-blocking ELISA, it was analyzed more in detail by using a series of GST-JUNV trNPs by ELISA (Fig. 4C). The reactivity of MAb E4-2 was normalized by dividing the OD_{405} value of MAb E4-2 by that of an anti-GST MAb. MAb E4-2 reacted with the GST-JUNV trNP containing the polypeptide KEVDRLMS (aa 72 to 79). The ELISA result was consistent with that of Western blotting (data not shown). The epitopes recognized by the MAbs are summarized in Fig. 5.

DISCUSSION

Detection of a viral Ag and/or the viral genome is crucial for rapid diagnosis of patients with hemorrhagic fever caused by South American arenaviruses, especially for patients in the acute phase. The application of reverse transcriptase PCR (RT-PCR) and TaqMan PCR for detection of the JUNV, MACV, and GTOV genomes has been reported (1, 11, 12, 27). Serological diagnosis is also useful for the diagnosis of AHF, especially in patients in the convalescent phase (7, 17, 19, 20, 26).

An Ag capture ELISA using a cocktail of MAbs against JUNV (25) was applied in an epidemiological study of rodents in Argentina (16). MAbs reactive with the NP of JUNV have been shown to cross-react with those of MACV and other nonpathogenic arenaviruses (25). In the present study, by using MAbs raised against the rNP of JUNV, we developed Ag capture ELISAs specific for JUNV and broadly reactive to human pathogenic New World arenaviruses.

The three MAbs to JUNV NP (designated C6-9, C11-12, and E4-2) reacted with the rNP of JUNV prepared using a baculovirus expression system by IgG ELISA and with rNP expressed in mammalian cells by IFA (Fig. 1 and Table 1). All Ag capture ELISAs using MAbs E4-2, C11-12, and C6-9 detected the rNP of JUNV (Fig. 2), suggesting that these ELISAs are useful tools for the diagnosis of AHF.

Interestingly, an Ag capture ELISA using MAb E4-2 detected the Ags of all of the pathogenic South American arenaviruses tested, in addition to that of JUNV (Fig. 2). IgG ELISA showed that the reactivity of MAb E4-2 with the rNP of JUNV was stronger than that with the rNPs of other South American arenaviruses (Fig. 1). The minimal length of the epitope required to be recognized by MAb E4-2 was 8 aa with the sequence KEVDRLMS (Fig. 4 and 5). However, the GST-JUNV trNP at aa 1 to 80 was more reactive than that at aa 1 to 79, which includes minimal epitope sequences, but was still less reactive than those at aa 72 to 564, 67 to 564, and 1 to 564 (Fig. 4). Even though we could not express GST-JUNV trNPs at aa 1 to 81 or more in *E. coli* because of their toxicity, it is possible that some additional amino acids at the C terminus of the minimal epitope are required for complete reaction with MAb E4-2. Actually, comparison of the amino acid sequences of NPs at positions 72 to 83 among South American arenaviruses showed that the amino acid differences with respect to JUNV were 1 aa for GTOV, 2 aa for MACV, 3 aa for SABV, and 5 aa for CHPV (Fig. 5), and these differences correlated well with the levels of reactivity of MAb E4-2 to the rNPs of the viruses (Fig. 1).

The Ag capture ELISA using MAb C11-12 also detected the Ags of all of the other pathogenic South American arenaviruses (Fig. 2). MAb C11-12 reacted with the rNPs of all of the pathogenic South American arenaviruses by IgG ELISA and IFA (Fig. 1 and Table 1). These results suggest that MAb C11-12 would be useful for detecting the Ags of all South American arenaviruses by Ag capture ELISA and IFA. Furthermore, the amino acid sequence (WTQSLR) of the epitope recognized by MAb C11-12 was located at the N terminus of the JUNV NP and was conserved among all of the pathogenic

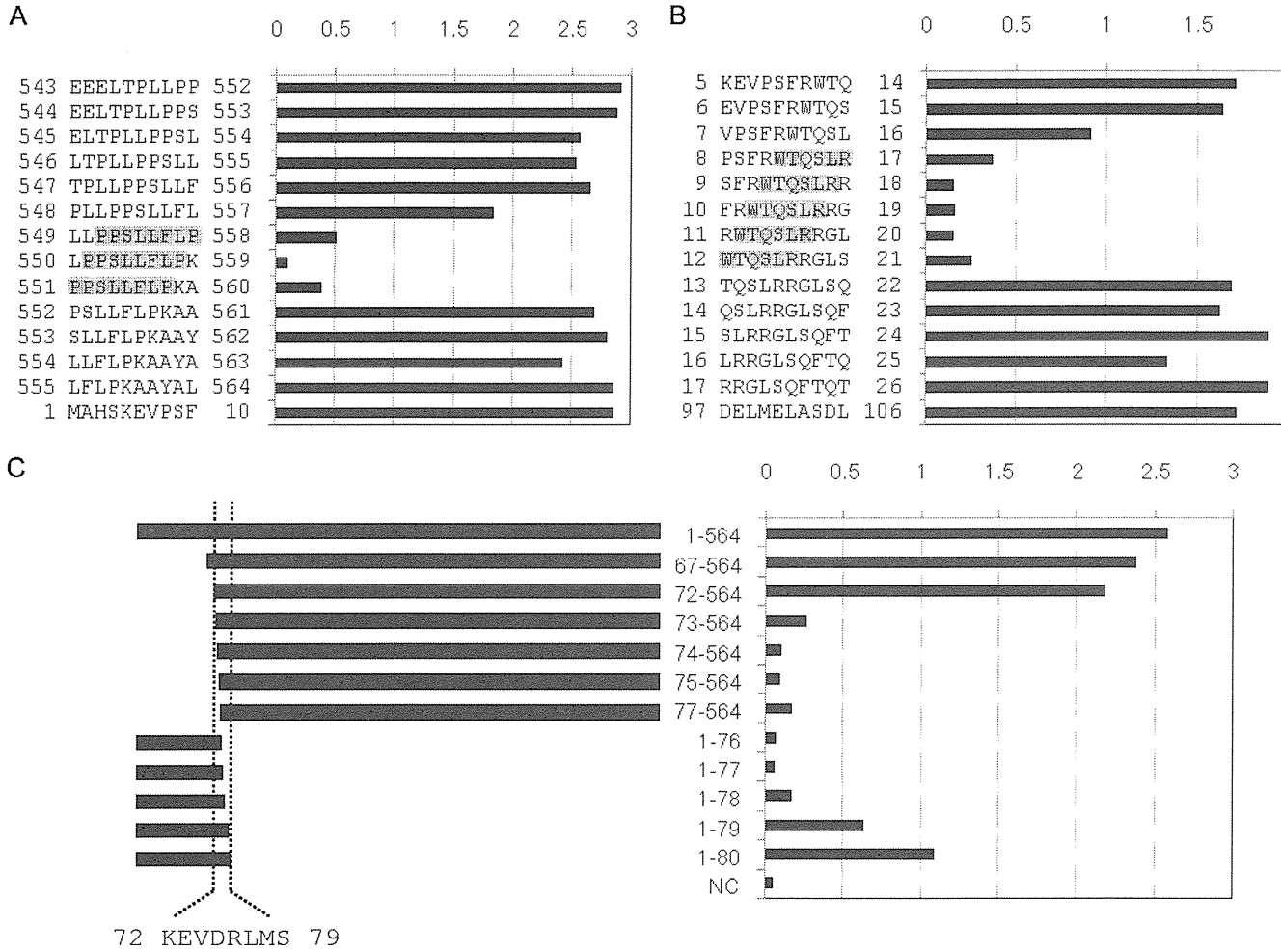

FIG. 4. Determination of the epitope on JUNV NP recognized by MAbs C6-9 (A), C11-12 (B), and E4-2 (C). (A, B) The ability of synthetic decapeptides to block the reactivity of MAbs to the JUNV rNP was examined by ELISA. The amino acid sequences and positions of synthetic peptides used in the assay are shown on the *y* axis. The synthetic peptides at aa 1 to 10 and 97 to 106 were used as negative control peptides for MAbs C6-9 and C11-12, respectively. Results indicate the OD_{405}. MAb C6-9 was confirmed to react with the 8 aa residues (PPSLLFLP) at positions 551 to 558, as represented by the shaded box (A). Similarly, MAb C11-12 was confirmed to react with the 6 aa residues (WTQSLR) at positions 12 to 17 (B). (C) The reactivity of MAb E4-2 with GST-tagged partial polypeptides of the JUNV NP was examined by ELISA. Schematic drawings of polypeptides of the JUNV NP are shown on the left, and the amino acid positions of the polypeptides are indicated on the *y* axis. NC represents the GST protein without any JUNV NP sequences. The reactivity of MAb E4-2 to each partial JUNV NP is indicated by the adjusted OD_{405}, which was calculated by dividing the OD_{405} of MAb E4-2 by that of the anti-GST MAb to the corresponding Ag. MAb E4-2 was confirmed to react with the 8 aa residues (KEVDRLMS) at positions 72 to 79, as indicated at the bottom.

South American arenavirus isolates so far deposited in GenBank (Fig. 5). However, slight differences in the sensitivity of detection of the NPs of the South American arenaviruses by Ag capture ELISA were observed. This may be due to the reactivity of the detector antibody, anti-JUNV NP rabbit serum, which was raised against the purified rNP of JUNV. Since the N-terminal region of the NPs recognized by MAbs C11-12 and E4-2 (aa 1 to 80) was relatively conserved among the NPs of South American arenaviruses, Ag capture ELISAs using MAbs C11-12 and E4-2 are considered to be useful for detecting most South American arenavirus isolates. Therefore, these Ag capture ELISAs may be applicable not only for the diagnosis of AHF but also for the diagnosis of BHF, VHF, and Brazilian hemorrhagic fever and may also be applicable for newly emerging viral hemorrhagic fevers caused by CHPV, although further study is needed.

On the other hand, the Ag capture ELISA using MAb C6-9 only detected JUNV Ag (Fig. 2). Furthermore, MAb C6-9 only reacted with the rNP of JUNV by IgG ELISA and IFA (Fig. 1 and Table 1). The amino acid sequence (PPSLLFLP) of the epitope recognized by MAb C6-9 was conserved among JUNV isolates so far deposited in GenBank (data not shown) but differed from those of other South American arenavirus isolates (Fig. 5). Since only the proline at position 552 in the epitope sequence is different in MACV, this proline is likely to be critical in the reaction of MAb C6-9. Therefore, the Ag capture ELISA using MAb C6-9 may detect most, if not all, JUNV isolates. Considering that the symptoms due to JUNV infection in humans are indistinguishable from those due to other South American arenaviruses, the Ag capture ELISA using MAb C6-9 may be a useful diagnostic tool, especially for AHF.

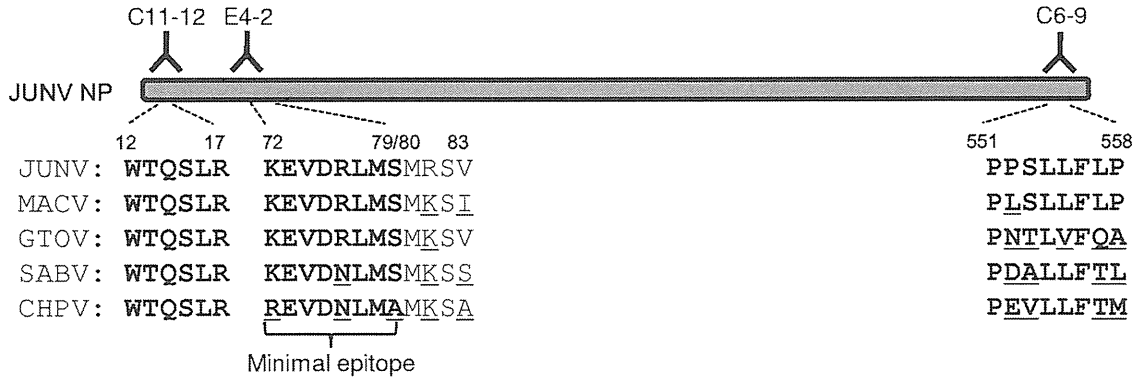

FIG. 5. Schematic representation of epitopes of the JUNV NP recognized by MAbs. The amino acid sequences of the epitopes of the JUNV NP recognized by MAbs C6-9, C11-12, and E4-2 are in boldface, and the amino acid positions are shown above the sequence. The amino acid sequences of the epitopes are compared to those of MACV, GTOV, SABV, and CHPV. The amino acid residues different from those of the JUNV NP are underlined. Because the amino acid sequence of the corresponding region is conserved among the strains of each virus species, a single sequence represents each virus species. The GenBank accession numbers for the S genes of JUNV are NC_005081, DQ272266, AY746353, AY619641, AY358023, D10072, U70802, U70803, and U70804. Those for the S genes of MACV are NC_005078, AY924208, AY924207, AY924206, AY924205, AY924204, AY924203, AY924202, AY571959, AY624355, AY619645, AY571904, AF485260, and AY129248. Those for GTOV are NC_005077, AY497548, AF485258, and AY129247. Those for SABV and CHPV are NC_006317 and NC_010562, respectively.

While the efficacy of newly developed Ag capture ELISAs in the diagnosis of viral hemorrhagic fever caused by South American arenaviruses was not evaluated by using serum samples from patients, it is generally accepted that an Ag capture ELISA is useful for the detection of viral Ags in blood and/or organ tissue specimens from patients in the acute phase.

The amino acid sequences of the epitope regions recognized by MAbs E4-2 and C6-9 were different from those of the corresponding region of LASV. On the other hand, the amino acid sequence (WTQSLR) of the epitope recognized by MAb C11-12 is the same in JUNV and LASV, even though the MAb failed to react to the LASV NP. However, aa 8 and 11 are proline and arginine in the NPs of South American arenaviruses while they are lysine/arginine and leucine in the LASV NP, respectively. Thus, it is possible that some amino acid sequence differences around the minimal epitope region affect the reaction with MAb C11-12.

In general, RT-PCR is more sensitive in detecting viruses in patients' specimens than is an Ag capture ELISA. Recently, a real-time RT-PCR has been established for the detection of all of the pathogenic South American arenaviruses, but it has not yet been applied for clinical specimens, so the possibility that it does not detect novel virus strains or species cannot be ruled out (27). Furthermore, arenaviruses are known to have high genetic variability, and false-negative results are occasionally obtained with some particular primer sets for RT-PCR. On the other hand, the Ag capture ELISAs established in the present study recognized highly conserved epitopes, suggesting that Ag capture ELISA may be useful for the diagnosis of suspected patients.

In conclusion, we developed Ag capture ELISA systems using newly produced MAbs against the JUNV NP and showed that JUNV Ag was detected specifically by the Ag capture ELISA using MAb C6-9. On the other hand, the Ags of all human pathogenic South American arenaviruses could be detected by an Ag capture ELISA using MAb C11-12 or E-4-2. The combined use of these Ag capture ELISAs in the present study may be useful for the diagnosis and differentiation of viral hemorrhagic fevers caused by South American arenavirus infections.

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Review:

Emerging and Reemerging Infection Threats to Society

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In societies where infectious disease outbreak potential is steadily rising, the heavy burden on society may adversely influence health status, social activities, and the economy. Influenza pandemics, for instance, threaten society internationally, while infectious outbreaks such as food-borne problems are usually limited to local or domestic levels. Risk factors in infectious outbreaks include destruction of the natural environment, increased international trade and travel, economic activity, lifestyle changes, medical practice, political instability including terrorist activity, and natural disasters. Recent emerging and reemerging infection did not originate naturally and spontaneously, but occurred through the above risk factors. Infectious outbreaks have increased in both size and frequency over those in the past. Risk factors for large-scale infectious emergence and reemergence threatening society should be scientifically analyzed and measures implemented in advance whenever possible.

Keywords: emerging infections, re-emerging infections, disasters, large-scale infections, bioterrorism

1. Introduction

The terms "emerging" and "reemerging" infection are being increasingly widely used everyone from medical circles to public forums due to the increasing number of emerging infections (1) representing major threats to health, the economy, and international trade, (2) involving high mortality, and (3) resulting from large-scale food-borne infection. Added to these is the bioterrorism threat from anthrax and variola virus causing smallpox [1–3]. One such apparent attack using anthrax occurred in the US in 2001 following the September 11, 2001, destruction of New York high-rises and damage to the Pentagon in Washington DC [3–5]. Natural disasters such as earthquakes, tsunamis, and hurricanes have left large numbers of residents in affected areas stuck living in unsanitary conditions for increasing periods of time. Such conditions expose populations to infectious diseases such as respiratory tract infections, gastrointestinal tract infections, and water-borne microbes. Climate change has become a major concern that is, of itself, expected to spread infectious diseases far beyond their traditional endemic areas.

Increased international travel, animal transport and trade, and destruction of the natural environment all present major risks triggering large-scale infectious outbreaks. Given this dire scenario, the sections that follow explore the emergence and reemergence of infectious diseases and suggest effective measures and strategies for minimizing their occurrence.

2. Recent Emerging and Reemerging Infections Seriously Threatening Society

The last three decades have seen an alarming increase in the threat from large-scale infectious disease, summarized in **Table 1**. Examples include acquired immunodeficiency syndrome (AIDS) due to the human immunodeficiency virus (HIV)[1], variant Creutzfeldt-Jakob disease (CJD) [6], Nipah encephalitis due to the Nipah virus [7, 8], severe acute respiratory syndrome (SARS) due to the SARS coronavirus [9, 10], highly pathogenic avian influenza virus H5N1[2], influenza A virus H1N1 infections [11]. The etiological agents of these infections are invariably zoonotic in origin – a situation that has, with subsequent occurrences throughout history produced "plagues" peculiar to their time and emergence, as indicated in **Fig. 1**.

1) AIDS, which was first identified in San Francisco, CA, US, in the early 1980s, was found to be caused by newly identified HIV [12] – an infection that is now a worldwide phenomenon infecting roughly 33 million people globally, one-third of whom live in sub-Saharan Africa[3]. Without proper antiretroviral treatment, HIV continues to be fatal and is mainly associated with sexual activity among relatively younger generations. As such, it has become a great community burden at the local, domestic, and international levels in the three decades since its discovery. HIV infects over 20% of the population in some sub-Saharan countries, wreaking havoc upon economies and cutting swaths through large numbers of younger populations.

1. http://www.who.int/hiv/data/en/
2. http://www.who.int/csr/disease/avian_influenza/en/
3. http://www.who.int/hiv/en/index.html

Table 1. Major recent emerging and reemerging infectious diseases.

Year	Pathogen	Disease	Reservoir animal	Amplifier animal	Comments
Early 1980s	HIV	AIDS	Chimpanzee?	None	20% of sub-Saharan region residents are infected with HIV.
Early 1990s	BSE prion	Variant CJD	Sheep	Cattle	European countries were hard hit.
1997 to date	Avian influenza A virus H5N1	Acute respiratory distress syndrome	Waterfowl	Chickens	South Asian countries were hard hit. Over 400 patients were reported, and mortality was high.
1998	Nipah virus	Nipah encephalitis	Fruit bats	Pigs	The largest outbreak of Nipah encephalitis occurred in Malaysia in 1998 and 1999
1999	West Nile virus	West Nile virus infection	Wild birds	None	Mosquito-borne infection. The Americas were free from West Nile virus until 1998.
2002-2003	SARS	SARS coronavirus infection	Bats?	Civet cats?	Over 8000 SARS cases with a mortality of 10% were reported. China was hard hit by SARS.
2006 to date	Chikungunya virus	Chikungunya virus infection	Human		Mosquito-borne infection
2009	Influenza A virus H1N1	Influenza	Waterfowl	Swine	

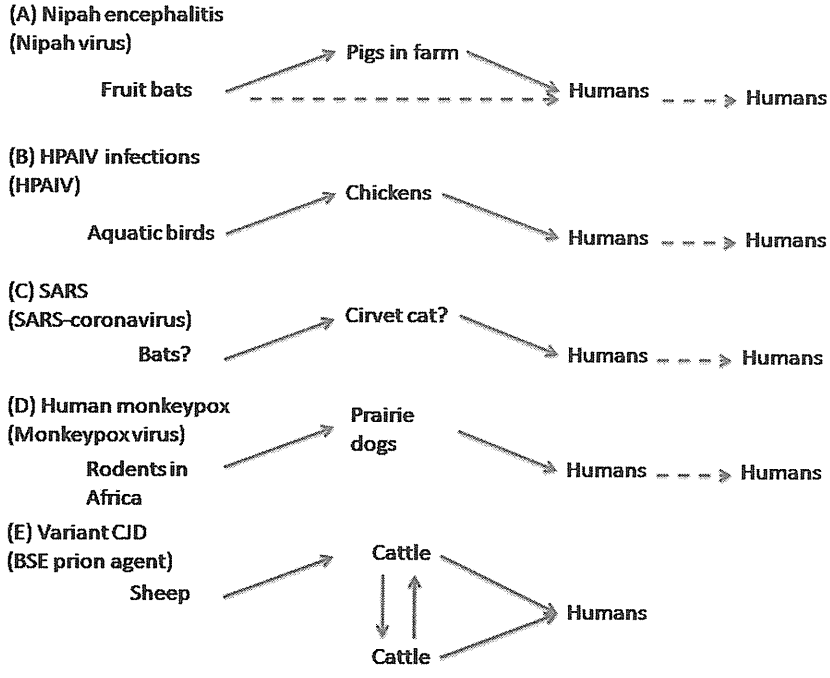

Fig. 1. Transmission of causative agents responsible for emerging and reemerging host-to-human infection. Note that such agents rarely infect humans directly but prceed from the host animal to human infection indirectly through amplification in another animal. Unbroken lines: Main transmission of etiological agents. Dotted lines: Transmission with lower incidence.

2) Variant Creutzfeldt-Jacob disease. Creutzfeldt-Jakob disease (CJD) is prototype human transmissible spongiform encephalopathy characterized by spongy degeneration of the brain with severe and fatal neurological signs and symptoms. CJD is relatively rare, caused by infection with an infectious protein known as a "prion" (CJD prion agent). Outbreaks of bovine spongiform encephalopathy (BSE), a bovine disease similar to CJD in humans, were first reported in the UK in 1986. BSE is an infectious,

neurodegenerative, fatal bovine brain disease. Since BSE was first discovered in 1986, it has increased in prevalence among cattle in the UK. In 1994, the first CJD patient, who had probably become infected with the BSE prion agent by eating contaminated meat, was identified [6, 13, 14]. CJD caused by eating meat contaminated by neuronal organs with the BSE prion agent is a variant of CJD (vCJD), with 130 vCJD cases reported, mainly in the UK and in France. To combat BSE and CJD outbreak, large numbers of UK cattle were slaughtered. Other measures taken to interrupt transmission of causative agents have included routine testing of beef or dairy cattle for the presence of the BSE prion agent, e.g., in Japan. BSE and CJD outbreaks placed a great burden on society economically, physically, and psychologically.

3) Nipah encephalitis. In 1998 and 1999, an outbreak of this mysterious high-mortality encephalitis was reported in Malaysia, then Singapore. A new virus, now known as Nipah virus, was isolated and confirmed to be the etiological agent for Nipah encephalitis [8, 15]. The Nipah virus reservoir was identified as species of fruit bat colonizing the region. Of the 265 victims infected, 105 died. An associated outbreak among slaughterhouse workers in Singapore in March 1999 led to 11 cases and 1 death [16]. The source of the Singapore outbreak was confirmed to be pigs infected with Nipah virus imported from Malaysia. Most patients were found to have been in contact with pigs suspected of harboring the virus either on pig farms or during shipping and slaughter. In transmission to humans, Nipah virus secreted by reservoir bats entered the pig population on affected farms, causing porcine respiratory illness. Nipah virus readily replicated in pigs and was heavily shed in excreta, exposing pig farm and slaughterhouse workers through close contact with infected pigs. In attempts to quell the outbreak, large numbers of pigs were slaughtered and Singapore banned pig imports from Malaysia.

4) SARS: A respiratory tract infection with high mortality reported in Guandong province in the southern People's Republic of China (PRC) in late 2002 spread mainly within the PRC and then worldwide. When a nosocomial outbreak was identified in a Hanoi, Vietnam, hospital in March 2003, it was finally recognized by the international community, including health authorities such as the World Health Organization (WHO), at which point the disease was named SARS. The outbreak originated and spread in southern China between late 2002 and June 2003. Human-to-human infection with SARS was confirmed, and the total number of patients identified with SARS reached approximately 8,000 with a mortality of about 10%. WHO issued a recommendation to the international community against travel to endemic areas such as the Hong Kong Special Administrative Region (SAR) and Guangdong province, and Toronto, Canada. Although the actual impact of the SARS outbreak on the global economy is difficult to calculate, its impact on the global community can be described as catastrophic.

5) Avian influenza. An outbreak of highly pathogenic avian influenza A virus H5N1 (HPAIV H5N1) in humans occurred in the Hong Kong SAR in 1997 [17]. Of 18 cases of human infection with HPAIV H5N1 identified in Hong Kong, 6 died [18]. Although the Hong Kong SAR outbreak was controlled, similar outbreaks later reemerged in other Southeast Asian countries such as Vietnam, Thailand, and Indonesia from 2003 on. HPAIV H5N1 eventually spread to central Asia, Europe, and Africa. As of April 23, 2009, 421 patients with HPAIV H5N1 infections have been reported, of whom 257 have died[4]. Because of the possible and potential pandemic threat due to HPAIV H5N1, preparedness has been strategically implemented locally, domestically, and internationally in developed countries, including Japan.

6) Influenza A. The Centers for Disease Control and Prevention (CDC), Atlanta, GA, reported that several patients with respiratory tract symptoms were infected with influenza virus type A (H1N1) in California and Texas in April 2009[5]. Soon after, the outbreak of influenza virus type A (H1N1) among residents in Mexico was reported. As of April 30, 2009, cases have been confirmed in Mexico, the US, Canada, Europe, Oceania, and other countries. This virus may well become the causative virus for the next pandemic in the very near future due to its high human-to-human transmission. In April 2009, WHO issued a warning regarding the high possibility of a pandemic outbreak and recommended preventive measures against the possibility of such outbreaks. This new virus may potentially place an enormous burden on society at all levels.

3. The Bioterrorism Threat

In March 1995, a cult called Aum Shinrikyo launched an attack using the bioweapon sarin in the central Tokyo subway system, killing 12 and making over 5,000 people ill [19]. This was preceded in June 1994 by a "practice run" when sarin was released by the same cult in Matsumoto, Nagano Prefecture, killing 8 and sickening over 200[6]. In 2001, bioterrorism-associated anthrax infections occurred in New York, Florida, and Washington, DC, US [3–5], not long after the September 11, 2001, commercial jetliner hijacking attacks destroying New York's World Trade Center and damaging Pentagon military headquarters in Arlington, VA, US.

Over 30 years have passed since the global eradica-

4. http://www.who.int/csr/disease/avian_influenza/country/cases_table_2009_04_23/en/index.html
5. Pro-Med-mail, Archive number: 20090422.1516
6. http://en.wikipedia.org/wiki/Matsumoto_incident

tion of the deadly infectious disease, smallpox, from which the world remains relatively free thanks large-scale and burdensome smallpox vaccination programs. Given that these programs were terminated over 3 decades ago, however, a large proportion of the global population has no immunity against the variola virus, and future smallpox outbreaks are now considered possible if, for example, a terrorist group reintroduces the variola virus. Some developed countries including Japan have initiated smallpox vaccine production and stockpiling against such threats [20, 21].

4. The Imported Infection Threat

Anxiety levels are being raised with the introduction of emerging infections from disease-epidemic regions to hitherto disease-free communities. As stated, the 2009 influenza virus H1N1 outbreak emerged in Mexico before spreading to the US, Canada, and Europe. By early May 2009, cases of infection spread rapidly to a number of disease-free countries, making this form of influenza virtually global in its dominion. Such emergence poses a great threat on many levels, from economic to psychological.

Cases worthy of consideration include the following:

1) West Nile virus infections in the US. Until 1998, the Americas were free of mosquito-borne West Nile virus infections, which were originally prevalent in Africa, Europe, and Asia, excluding the Far East. An encephalitis caused by West Nile virus was reported in New York in 1999 [22] and, since then, such infections have emerged each summer, spreading progressively across the US and into neighboring nations.

2) Chikungunya virus infections in Italy. Chikungunya fever, usually a self-limiting febrile illness, has emerged in the form of large outbreaks in Africa, in Indian Ocean island countries, and in South Asia [23]. Italy was Chikungunya fever-free until 2007, when an epidemic broke out there with over 200 patients with Chikungunya fever [24]. Chikungunya virus is transmitted during infection between humans and host mosquitoes. Epidemiological and virological studies showed the outbreak to have been caused by a single patient from India who introduced the Chikungunya virus into the local community through vector mosquitoes.

3) Human monkeypox in the US. Monkeypox virus infection in humans, known as human monkeypox, is clinically similar to smallpox, and the etiological agent is the monkeypox virus, which, together with the variola virus, belongs to genus *Orthopoxvirus*, family *Poxviridae*. Hosts are species of rodents native to central and western Africa, indicating that human monkeypox was once prevalent only in these regions. An outbreak occurred in the US in 2003 [25] due to African dormice and Gambian giant rats imported from Accra, Ghana, to the US as pets, shedding the virus in their excretions. Monkeypox virus then infected prairie dog colonies, whose inhabitants were also sold as pets. People coming in contact with infected prairie dogs contracted human monkeypox. In this outbreak, with over 60 patients, the similarity of clinical human monkeypox and smallpox symptoms panicked the general public by raising talk of possible bioterrorist attacks with variola virus early in the outbreak.

5. Disasters and Infections

Natural disasters such as earthquakes, hurricanes, cyclones and typhoons, tsunamis, volcanic eruptions, floods, and drought are considered major risk factors for infectious disease emergence among displaced refugees, as indicated in **Table 2**. Such risk depends on the disaster type, size, and level, the local climate and environment, the displaced population, and the level of vaccine immunity.

Water-borne infections commonly occur following earthquakes, tsunamis, and floods, caused mainly by *Vibrio cholerae*, enterotoxigenic *Escherichia coli*, *Salmonella enterica*, *Cryptosporidium parvum* leading to diarrheal diseases [26–30], hepatitis A and E viruses causing hepatitis, and bacterial leptospirosis transmitted by direct contact with water contaminated by leptospires. Contaminated water, of course, facilitates the spread of such infectious agents [31, 32].

Outbreaks of measles and other vaccine-preventable diseases, *Neisseria* meningitis, and viral respiratory tract infections such as influenza are often reported among refugees forced to live in unhygienic, and crowded conditions following natural disasters [33–35]. In meteorological events, vector-borne outbreaks occur due to dengue virus and malaria [36, 37] usually mosquito-associated. Changes in the environment favoring mosquito breeding are the suspected cause.

6. Political Instability and Infections

The southern African nation of Zimbabwe mired in political and economic crises with large parts of its population living drastically disrupted lives, compounded by inadequate hygiene. Cholera, an acute diarrheal infection caused by *Vibrio cholerae* mostly spread through contaminated water and food and poor hygiene, kills even healthy adults quickly through severe dehydration, making the prevention of dehydration the key to preventing cholera fatalities. As of March 17, 2009, however, 91,164 cases with 4,037 deaths were reported during an outbreak beginning in August 2008[7]. A cholera outbreak was also reported in politically and economically unstable war-plagued Iraq in 2007[8,9]. The situation in these

7. http://www.who.int/wer/2009/wer8414.pdf
8. http://whqlibdoc.who.int/wer/WHO_WER_2007/82_357-360(no41).pdf
9. http://www.who.int/wer/2008/wer8341.pdf

Table 2. Large-scale infectious diseases associated with natural disasters, economic activity, and medical practices.

Events		Infectious disease				
		Viral	Bacterial	Fungal	Parasitic	Others
Natural disasters		ARI, Hepatitis due to HAV and HEV, measles, dengue	Salmonellosis, Cholera, E. coli infections, Leptospirosis	Coccidioides	Malaria	
Economic activities	Chicken farming	Avian influenza virus infection	Salmonellosis			
	Pig farming	Nipah encephalitis				
	Cattle farming					vCJD
	Mass production of food	Norovirus gastroenteritis	Staphylococcal infections, enterohemorrhagic *E. coli* infections including HUS, Shigellosis, Botulism, Listeriosis, Campylobacter infections			
Medical practices	Treatment with blood products	AIDS, hepatitis due to HBV and/or HCV				
	Dura mater transplantation					CJD

two countries plays up the potentially positive role that politics could play in preventing such outbreaks.

7. Large-Scale Food-Associated Infections

Large-scale food-borne infections pose great physiological and psychological burdens on communities. The fact that most food is mass-produced and widely distributed in developed countries has led to many large-scale food-borne infections affecting large populations in large numbers of communities and engendering high economic loss.

A large-scale food poisoning due to *Staphylococcus aureus* occurred in Japan's western Kansai region in 2000, triggering over 10,000 cases and resulting in the responsible enterprise being shuttered permanently. Another large-scale food-borne diarrheal outbreak due to hemorrhagic *Escherichia coli O157/H7* occurred in the same area in 1996 and assumed due to *E. coli* O157/H7 found in *kaiware* radish sprouts, a severe infection leading to hemolytic uremic syndrome (HUS) with renal failure. The actual source was never identified, despite 8,000 cases and three deaths. A similar outbreak due to *E. coli O157/H7* reported in the US in 2006[10] affected some 400 persons in several states across the country. The outbreak was thought to be due to a California Taco Bell product distributed to restaurants nationwide.

Variant CJD in the early 1990s in the UK, as stated above, resulted in massive cattle culls that put some farmers out of business and caused tremendous domestic and international economic and other burdens impacting significantly and negatively on the global economy.

8. Medical Practices and Large-Scale Infections

Potential iatrogenic (physician-induced) outbreaks are also an important issue affecting communities. Blood-borne infections such as HIV, hepatitis B, and hepatitis C are prime examples resulting when patients are treated with contaminated blood products. Outbreaks of iatrogenic infections inflict great stress on patients nationwide treated with blood products produced before blood product contamination was halted by heating. Hepatitis B and C carry the related risk of hepatic cirrhosis and hepatocellular carcinoma, making them serious problems indeed.

Another example is a large-scale iatrogenic CJD outbreak occurring in Japan in the late 1980s when patients were infected with the CJD prion agent through dura mater transplantation in neurosurgical procedures. The contamination source Lyodura™, produced by a German pharmaceutical firm whose products were produced using dura mater from a person infected with the CJD prion agent. Over 100 cases of associated CJD resulted. Although dura-mater transplantation-associated CJD was reported in Japan in the early 1990s, no preventive measures were implemented, making Japan the "leader" in such infections. In the US, in contrast, only one case was reported in the US, in 1987 [38], involving a 28-year-old woman – exceptionally young among CJD patients – who had undergone Lyodura™ transplantation. Authorities responsible for biological product screening and control, including the US Food and Drug Administration (FDA) and the Centers for Disease Control and Prevention (CDC), considered that the patient may have been infected with the CJD prion agent and issued a warning

10. Pro-Med mail: Archive number: 20061213.3505

against neurosurgical Lyodura™ use, thus underscoring the importance of health authorities in implementing appropriate, timely preventive measures early on in an outbreak.

9. Preparedness

Preparedness should focus on strategies to reduce incidence, minimize the burden on the community, and reduce panic in the population. Preparedness includes clarifying how these infections occur, the reasons behind their emergence, and their consequences.

Preparedness also includes enhancing research, increasing the number of specialists in government and public sectors working on these problems, increasing global cooperation through internationally recognized organizations such as the United Nations and WHO, enhancing timely information-sharing among responsible agencies, and disclosing information appropriately to the public.

Disaster threats associated with large-scale infectious outbreaks depend on local regional circumstances, such as Japan's vulnerability to major earthquakes. As stated, Japan has experienced relatively large-scale food-associated infectious outbreaks, including iatrogenic outbreaks of HIV and CJD. Emerging viral infections, such as hemorrhagic fevers and Nipah encephalitis, however, tend to occur in developing rather than developed nations, making it vital to analyze the risk of occurrence of emerging infections threatening communities at local, domestic, and international levels.

Once a large-scale outbreak occurs, measures should be implemented to combat it as soon as possible, focusing on identifying etiological agents and clarifying outbreaks, which underscores the importance of risk communication. The public should be kept up-to-date and informed of exactly what is happening and what actions to take through well-implemented information dispensation, by governmental, scientific, and media sectors.

10. Conclusions

Infectious disease outbreaks burdening the global community have been reviewed and found to be closely associated with human activity. Environmental destruction, economic activities such as dairy husbandry in association with poor hygiene, medical practice, international travel, international trade in food and animals, and bioterrorism are the major triggers behind such outbreaks. International cooperation needs to be promoted if these threats to humanity are to be controlled and, eventually, prevented.

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Diagnostic Systems for Viral Hemorrhagic Fevers and Emerging Viral Infections Prepared in the National Institute of Infectious Diseases

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Given the real possibility of hemorrhagic fever viruses such as ebola (EBOV), Marburg (MARV), Crimean-Congo hemorrhagic fever (CCHFV), and Lassa (LASV) viruses being introduced into virus-free nations such as Japan, the need arises for concomitant diagnostics even where such diseases are not endemic. Hemorrhagic fever viruses classified as biosafety level-4 (BSL-4) pathogens can only be manipulated in BSL-4 laboratories, making it difficult for nations such as Japan, having no BSL-4 laboratories, to develop required diagnostic assays. To circumvent this problem, diagnostic assays with recombinant viral antigens have been developed at the National Institute of Infectious Diseases, Tokyo, Japan (NIID). Diagnostics such as enzyme immunoassays for detecting viral hemorrhagic antibodies and antigens were developed using recombinant nucleoproteins (rNPs) of EBOV, MARV, CCHFV, and LASV for use as antigens. Immunoglobulin-G (IgG)-linked immunosorbent assay (ELISA) and indirect immunofluorescence assay using rNPs were confirmed to be highly sensitive and specific in detecting these antibodies. Sandwich antigen (Ag) capture ELISA was also developed for detecting these antigens. The sections that follow detail diagnostics developed at the NIID.

Keywords: viral hemorrhagic fevers, diagnosis, biosafety level 4-pathogens, preparedness, recombinant nucleoproteins

1. Introduction

Viral hemorrhagic fevers include ebola hemorrhagic fever (EHF), caused by the Ebolavirus (EBOV), and Marburg HF (MHF), caused by the Marburgvirus (MARV), which belong to the *Filoviridae* family and are responsible for mortality of 80-90% in Africa [1-4]. EBOV consists of five species – Zaire EBOV first isolated in the Democratic Republic of Congo, Sudan EBOV isolated in Sudan, Ivory Coast EBOV isolated where its name indicates, Bundibugyo EBOV first isolated in Uganda, and Reston EBOV first isolated in the Philippines [4]. MARV consists of one species, Lake Victoria MARV. Lassa fever (LF), a viral hemorrhagic fever caused by the Lassa virus (LASV), is an Old World arenavirus occurring in such west Africa nations as Guinea, Sierra Leone, and Nigeria [5-10], where it infects tens of thousands annually and kills hundreds to thousands endemically from central to west Africa [11]. Argentine hemorrhagic fever, caused by the New World arenavirus, Junin (JUNV), is endemic to Argentina. Similar New World hemorrhagic fevers reported in Bolivia, Brazil, and Venezuela [11] are called South American hemorrhagic fever (SAHF). Crimean-Congo hemorrhagic fever (CCHF), an acute viral hemorrhagic fever, accounts for mortality of 30% [12]. CCHF virus (CCHFV) is a member of the family *Bunyaviridae*, genus *Nairovirus* [18]. EHF, MHF, LF, CCHF, and SAHF are classified as category 1 infectious diseases under Japan's Infectious Disease Control Law, enacted in 1998 and amended a decade later.

Increased international travel and animal trade present enormous potential for viral hemorrhagic fever outbreaks outside endemic areas in previously virus-free areas, as shown by the case of a woman infected with MARV during her Uganda stay[1] who died of it in the Netherlands in 2008. In another example, over 20 LF cases have been reported outside endemic regions in the United States (US), Canada, Europe, and Japan [13-19].

EBOV, MARV, CCHFV, LASV, and SAHF viruses are internationally and domestically categorized as biosafety-level 4 (BSL-4) pathogens whose manipulation requires a BSL-4 laboratory designed for work with dangerous and exotic agents posing a high laboratory infection risk and life-threatening disease. Japan's only currently operating facility is a "glove-box" BSL-4 setup implemented 25 years ago.

To circumvent this situation, recombinant viral antigens such as recombinant nucleocapsid proteins have been substituted for infectious hemorrhagic fever viruses for development of antibody detection systems. Antigen detection has also been developed, as detailed in the review of diagnostics for hemorrhagic fevers developed at the NIID that follows.

1. ProMed-mail, Archive number 20080711.2115

Table 1. EHF and MHF outbreaks due to virus importation to virus-free countries.

Virus	Country	Year	Patient Death	Description
LV MARV[a]	Germany and Serbia/Herzegovina	1967	7/31	MARV responsible for the outbreak originated in a monkey imported from Uganda.
LV MARV	Zimbabwe and South Africa	1975	1/3	The index case was infected with MARV in Zimbabwe. Nosocomial infection occurred in a South African hospital.
LV MARV	US	2008	0/1	The patient was infected with MARV in Uganda and diagnosed with MHF by serological diagnosis.
LV MARV	Netherlands	2008	1/1	The patient was infected with MARV in Uganda.
Reston EBOV	US	1989/1990	0/4	EHF outbreak in nonhuman primates imported to the US from the Philippines. Four asymptomatic persons were confirmed infected with Reston EBOV.
Reston EBOV	Italy	1992	0/0	EHF outbreak in nonhuman primates imported to Italy from the Philippines.
Zaire EBOV	Gabon and South Africa	1996	45/60	Nosocomial infection occurred in a hospital in which a nurse caring for a doctor transferred from Gabon died.

a: LV MARV: Lake Victoria MARV.

b: DRC: Democratic Republic of the Congo.

2. Viral Hemorrhagic Fevers

2.1. Ebola and Marburg Hemorrhagic Fevers

EBOV and MARV, which cause EHF and MHF, belong to the family Filoviridae. EBOV and MARV are classified into the genera Ebolavirus and Marburgvirus. Genus Ebolavirus consists of 5 viral subspecies – Zaire, Sudan, Ivory Coast, Bundibugyo, and Reston EBOVs – while genus Marburgvirus consists of the single viral species Lake Victoria MARV. Natural hosts of EBOV and MARV are thought to be fruits bats in west and central Africa [20, 21]. EHF and MHF are two of the most severe forms of viral hemorrhagic fever, whose human victims are usually infected through close contact with contaminated blood, tissue, and/or excretions from viremic animals, such as nonhuman primates, or from human patients. Infected individuals abruptly develop flu-like symptoms characterized by fever, chills, malaise, and myalgia. They then usually develop signs and symptoms of systemic involvement such as prostration and gastrointestinal problems including anorexia, nausea, vomiting, abdominal pain, diarrhea; respiratory problems including chest pain, shortness of breath, and cough; vascular problems including conjunctival injection, postural hypotension, and edema; and neurological problems including headache, mental confusion, and coma. Bleeding manifests as petechiae and ecchymosis (subcutaneous bleeding), uncontrolled oozing from venipuncture sites and gingiva, mucosal hemorrhaging, and bloody diarrhea. In later stages, the general condition of victims deteriorates due to multiorgan failure, including disseminated intravascular coagulopathy (DIC), resulting in death [22-25].

The first recognized EHF outbreaks occurred in the Democratic Republic of Congo (formerly Zaire) and Sudan in 1976 [26-29]. After EBOV was discovered in 1976, several African nations were struck by EHF outbreaks caused by one of the four known human-pathogenic EBOV species – Zaire EBOV, Sudan EBOV, Ivory Coast EBOV, and Bundibugyo EBOV [30-39]. EHF outbreaks caused by Reston EBOV, occurred among cynomolgus macaques imported from the Philippines to the US in 1989 [40]. Reston EBOV was also introduced to the US in 1989, 1990, and 1996, and to Italy in 1992, through the same importation route [40-43]. It should be noted that the EHF outbreak incidence is increasing and that the number of EHF patients is rising.

The first documented MHF outbreak occurred in Germany, followed by one in Yugoslavia, in 1967 [44]. Technicians and scientists contracted MHF through the manipulation of tissue specimens collected from African green monkeys imported from Uganda and believed to already have been infected with MARV before importation. Three sporadic MHF cases were reported in Zimbabwe (1975) and Kenya (1980 and 1987) [31, 45-47]. From 1998 to 1999, a large outbreak occurred in the Democratic Republic of Congo [48]. The largest MHF outbreak occurred in Uige Province, Angola, in 2004, with 374 cases reported and mortality exceeding 88% as of August 24, 2005[2]. In 2008, a fatal MHF case was reported in the Netherlands[3] in which the patient contracted MHF in Uganda, showed symptoms after her return to the Netherlands, and eventually died.

Several cases of EHF and MHF outbreaks in nonendemic regions have also be imported, as detailed in **Table 1**, due to nosocomial and familial infection.

2.2. Crimean-Congo Hemorrhagic Fever

Humans acquire CCHFV infection primarily through tick (genus *Hyalomma*) bites or contact with fresh meat or blood from slaughtered viremic animals, including sheep, cattle, and goats. CCHF symptoms range in severity from fever alone or fever with flu-like symptoms to hemorrhage

2. http://www.who.int/csr/don/2005_08_24/en/index.html
3. ProMed-mail, Archive number 20080711.2115

Table 2. Recombinant antigen-based antibody detection developed at the NIID.

Method	Antigen origin	Antigen (amino acid position)[a]	Expression of recombinant protein	Sensitivity	Specificity	Reference
ELISA	Zaire EBOV	rNP	Recombinant baculovirus	13/14	50/51	[51]
		Truncated (361-739) rNP	Transformation of *E. coli* with expression vector	13/14	50/51	[51]
	Reston EBOV	Truncated (360-739) rNP	Transformation of *E. coli* with expression vector	10/10	72/72	[55]
	LV-MARV[d]	Truncated (341-695) rNP	Transformation of *E. coli* with expression vector	3/3	62/62	[51]
	CCHFV Chinese strain 8402	rNP	Recombinant baculovirus	13/14	107/109	[52]
	LASV	rNP	Recombinant baculovirus	4/4	94/96	[54]
IIFA	Zaire EBOV	rNP	Infection of HeLa cells with recombinant baculovirus	14/14	47/48	[59]
	Reston EBOV	rNP	Transfection of HeLa cells with expression vector	16/16	96/96	[58]
	LV MARV	rNP	Transfection of HeLa cells with expression vector	NT[e]	NT	Not reported
	CCHFV	rNP	Transfection of HeLa cells with expression vector	13/13	108/108	[57]
	LASV	rNP	Transfection of HeLa cells with expression vector	4/4	96/96	[54]

a: The amino acid position is counted from the translational initiation codon for each protein.

b: "Sensitivity" is defined as the number of samples reacting positively reaction in antibody detection divided by the number of positive controls.

c: "Specificity" is defined as the number of samples reacting negatively in antibody detection divided by the number of negative controls.

d: LV-MARV: Lake Victoria MARV.

E: NT: "Not tested". One serum sample collected from a MHF patient reacted positively, however, while 96 Japanese subjects reacted negatively.

with multiple-organ failure resulting in death. All patients develop fever and joint pain, and orbital pain, backache, and headache are common symptoms. Severe cases may show elevated liver enzymes and oliguria (decreased urine output) assumed associated with renal failure due directly to CCHFV or indirectly to hypovolemic (severe blood and fluid loss) shock.

CCHF is endemic to Africa, Eastern Europe, the Middle East, and Central and Southern Asia [49], and victims are believed to far exceed the number reported, since the disease usually occurs in remote areas. The first case of CCHF, reported in Turkey in 2002, has now totaled over 1,000. Two CCHF cases imported from Africa to Europe have been reported – one from Zimbabwe to the UK[4] and the other from Senegal to France[5].

2.3. Lassa and South American Hemorrhagic Fevers

Many cases of LF, caused by the Old World arenavirus LASV, have occurred in western Africa. Humans become infected through contact with infected excreta, tissue, or blood from the peridomestic rodent *Mastomys natalensis*, the LASV reservoir host [50]. LASV is transmitted among humans via mucosal and cutaneous contact or nosocomial contamination [7]. Argentine hemorrhagic fever caused by JUNV infection is endemic to Argentina. The pathophysiology of SAHF, including Argentine hemorrhagic fever, is similar to that of LF. The reservoirs of these arenaviruses are rodents in endemic areas and local inhabitants share their environment with these rodents, making prevention of arenavirus infection difficult. Among residents of Ghana and Nigeria, 15-20% showed a positive reaction to IgG antibody to LASV, indicating the common occurrence of LF there (our unpublished data). The number of LF cases imported from endemic to LF-free regions, including Japan, is the highest among all viral hemorrhagic fevers discussed thus far.

3. Diagnostic Assays Developed at the NIID

3.1. Recombinant NP-Based Diagnostics

3.1.1. ELISA

The recombinant nucleocapsid proteins (rNPs) of Zaire and Reston EBOV, Lake Victoria MARV, CCHFV, LASV, and JUNV were expressed using baculovirus as fusion proteins with a 6XHis-tag on the N-terminus [51-55]. rNPs thus expressed were purified and used as antigens

4. ProMed-mail, Archive number 19980109.0062
5. ProMed-mail, Archive number 20041125.3152

Table 3. Antigen-capture ELISAs developed for HFV antigens, including target proteins, capture and detector antibodies, and EBOV and MARV species reacting with individual capture antibodies.

Target protein	Capture antibody	Recognition site of capture antibody	Detector antibody	Samples	Comments	References
Zaire EBOV NP	MAb, 3-3D, to Zaire EBOV	Carboxy-terminal region of Zaire EBOV NP	Rabbit serum raised to Zaire EBOV rNP	Blood, serum, tissue	MAb, 3-3D, reacts with NPs of Zaire, Sudan, Reston EBOVs and possibly with NP of Ivory Coast EBOV.	[60]
Reston EBOV NP	MAb, Res2-6C8, to Reston EBOV NP	Carboxy-terminal region of Reston EBOV NP	Rabbit serum raised to Reston EBOV rNP	Blood, serum, tissue, and other body fluids	MAb, Res2-6C8, specifically reacts with NP of Reston EBOV.	[61]
	MAb, Res2-1D8, to Reston EBOV NP	Carboxy-terminal region of Reston EBOV NP	Rabbit serum raised to Reston EBOV rNP	Blood, serum, tissue, other body fluids	MAb, Res2-1D8, specifically reacts with NP of Reston EBOV.	[61]
LV MARV[c] NP	MAb, 2A7, to MARV NP	Carboxy-terminal region of MARV NP (from amino acid residue 632 to 645)	Rabbit serum raised to MARV rNP	Blood, serum, tissue, other body fluids	MAb, 2A7, reacts with MARV NP but not with NPs of EBOV.	[56, 62]
	MAb, 2H6, to MARV NP	Carboxy-terminal region of MARV NP (from amino acid residue 643 to 695)	Rabbit serum raised to MARV rNP	Blood, serum, tissue, other body fluids	MAb, 2H6, reacts with MARV NP but not with NPs of EBOV.	[56, 62]
CCHFV NP	MAb, 1B7, to CCHFV NP	Central region within CCHFV NP (from amino acid residue 201 to 306)	Rabbit serum raised to CCHFV rNP	Blood, serum, tissue, other body fluids	Efficacy in diagnosis of CCHF was evaluated	[63]
LASV NP	MAb, 4A5 to LASV NP	Five-amino acid residues positioned from 439 to 443	Rabbit serum raised to LASV rNP	Blood, serum, tissue, other body fluids	MAb, 4A5, reacts with NP of LASV but not NPs of LCMV[a] or JUNV[b].	[54]

a: LCMV: lymphocytic choriomeningitis virus, an Old World arenavirus.

b: JUNV: Junin virus, a New World arenavirus.

for IgG-ELISA and IgM-capture ELISA. As shown in **Table 2**, all IgG-ELISA systems using these rNP antigens were confirmed to be highly sensitive and specific.

The carboxy-terminal half of Zaire EBOV and MARV NPs were strongly antigenic [51]. The carboxy-terminal half of MARV and EBOV rNPs were therefore expressed, purified, and used as antigens in IgG-ELISA. These truncated rNP-based ELISA systems were also confirmed to be highly sensitive and specific in detecting antibodies. Similarly, the carboxy-terminal half of the Reston EBOV rNP showed similar antigenicity as the full-length NP of Reston EBOV and was used as the antigen in the IgG-ELISA format [55].

IgM-capture ELISA using CCHFV rNP as an antigen was confirmed to be efficacious in diagnosing CCHF in the early disease phase [56]. IgM-capture ELISA with VHF virus rNPs might be as efficacious in diagnosing hemorrhagic fever viruses as it was in CCHF.

The LASV rNP was also used as an antigen to detect antibodies to LASV [54]. A seroepidemiological study indicated that 20% of Nigerian people reacted positively to LASV rNP-based IgG ELISA, suggesting that one-fifth of the Nigerian population have a history of LASV infection (unpublished data).

3.1.2. Indirect Immunofluorescent Assay

Indirect immunofluorescent assay (IIFA) was developed using mammalian HeLa cells in which Zaire EBOV, Reston EBOV, Lake Victoria MARV, CCHFV, or LASV rNP was expressed [54, 57, 58] by cell transfection with expression vector pKS336 encoding NP genes [57]. In IIFA developed for Zaire EBOV, the Zaire EBOV rNP was expressed in HeLa cells by abortively infecting them with the recombinant baculovirus, in which the polyhedrin promoter was replaced with human cytomegalovirus immediate early (IE) promoter, then inserted with the Zaire EBOV NP gene [59]. Zaire EBOV rNP expression was controlled by the IE promoter. These hemorrhagic fever virus rNP-based IIFAs were useful in detecting antibodies both highly sensitively and specifically.

3.1.3. Antibody Detection ELISA

In fatal cases of viral hemorrhagic fever, patients usually die before antibody response, making it vital to detect the antigen to diagnose infection as early as possible. High titers of infectious hemorrhagic fever viruses are present in the blood and tissues early on in illness. Antigen-capture ELISA for detecting Zaire EBOV [60], Reston EBOV [61], MARV [56, 62], CCHFV [63, 79], and LASV [54] antigens has also been developed at the NIID using NP target proteins. Monoclonal antibodies to rNPs of these viruses were produced and used as capture antibodies. Polyclonal antibodies are raised in rabbits immunized with rNPs and polyclonal antibodies are then used as detection antibodies. Antigen-capture ELISA is summarized in **Table 3**. Although monoclonal antibodies used in antigen-capture ELISA were produced by immunizing mice with rNPs, NP-capture ELISA de-

tected both the rNPs of these viruses and the authentic virus NPs. Antigen-capture ELISAs were developed to detect NPs of Zaire, Sudan, and Reston EBOV [60], that of Reston EBOV alone [61], and that of MARV alone [56]. MARV NP-capture ELISA detected the authentic MARV NP [62], and MARV antigen-capture ELISA showed similar detection for the MARV antigen as MARV genome amplification by conventional reverse transcription-polymerase chain reaction (RT-PCR).

An evaluation of CCHFV antigen detection ELISA efficacy in diagnosing CCHF [63] showed that although nested RT-PCR sensitivity for diagnosing CCHF was higher than that of antigen-capture ELISA, CCHFV antigen-capture ELISA was efficacious particularly in diagnosing CCHF in patients prior to antibody response onset. The presence of antibodies to CCHFV in serum decreased sensitivity in detecting antigens in antigen-capture ELISA [63].

LASV antigen-capture ELISA was developed. Unfortunately, antigen-capture ELISA efficacy in diagnosing LF in patients has not yet been evaluated, but ELISA was confirmed to have sensitivity in detecting LASV antigen in serum samples collected from hamsters experimentally infected with LASV similar to LASV genome detection by conventional RT-PCR [54].

3.1.4. Other Diagnostic Assays

Our laboratory also works with diagnostics such as real-time quantitative RT-PCR. Histological techniques, including antigen detection by immunohistochemical analysis, are sensitive particularly in postmortem diagnosis [64]. Virus identification and detection are also available for blood and tissue specimens collected from patients.

4. Summary

Viral hemorrhagic fever diagnostic developed at the NIID have been reviewed and found to be valuable tools for use both in countries free from viral hemorrhagic fevers and in those where such fevers are endemic.

EHF caused by the newly identified EBOV Bundibugyo ebolavirus was reported in Uganda [4], and severe VHF infections caused by previously unknown arenaviruses have been reported in Zambia and South Africa (ProMed-mail, Archive number 20081028.3409). These facts indicate the need to continue developing, modifying, and improving VHF diagnostics.

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Review:

Imported Rabies Cases and Preparedness for Rabies in Japan

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Rabies has been causing some 55,000 annual deaths worldwide, mostly in the developing countries in Asia and Africa. At present, safe and effective post-exposure prophylaxis (PEP) has been established and applied to more than 10 million persons each year. Nevertheless, fatal infection of human rabies imported or from wild animals has not been eradicated even in developed countries. In Japan, two imported cases occurred in 2006, the first such cases in 36 years. It is necessary to inform the public about rabies, in particular for overseas tourists, to establish the prompt and appropriate PEP systems, and to improve vaccine supply systems in order to prevent the recurrence of rabies cases in Japan.

Keywords: rabies, zoonosis, post-exposure prophylaxis (PEP)

1. Introduction

A rabies case occurred in Kyoto City in November 2006 for the first time in 36 years in Japan. The patient traveled to the Philippines, where he was bitten by a dog, in August of the same year. Another case occurred a week later in Yokohama City. The patient was bitten by a dog also in the Philippines [1, 2]. Further incidences were apprehended, because two cases were recorded one after another for the first time in 36 years. Fortunately, no further cases have been reported thereafter. Although it was considered that increased outbreaks of rabies at that time in the Philippines brought the rabies incidences in Japan, no epidemic explosion in the Philippines has been reported. These two cases are considered to be accidental coincidence. The cases were shocking for the Japanese people to recognize anew awfulness of rabies, which had been forgotten for a long time. These cases have shed light on problems involved in the countermeasures in Japan against rabies in consideration that the above cases could be prevented if the two tourists had been treated by appropriate post-exposure prophylaxis (PEP).

PEP is an established treatment intended to prevent rabies encephalitis by conferring immunity to a person with a vaccine during the incubation period after he is exposed to rabies virus by being bitten by a rabid animal and before the clinical symptoms begin. More than 10 million people are treated by PEP worldwide each year. It is essential for the exposed persons to be vaccinated as soon as possible. The basic inoculation schedule recommended by the World Health Organization (WHO) is as follows; an intramuscular inoculation of rabies vaccine on the first day, 3rd, 7th, 14th and 28th days (Essen treatment). In Japan, an exposed person will be further vaccinated on the 90th day. WHO also recommends the application of several inoculation procedures to save amount of vaccines used [3].

Rabies had been endemic in Japan for a long time, and the epidemics were recorded in the Edo Period (1603-1867). Annually more than 100 patients died of rabies in the chaotic times after the Great Kanto Earthquake in 1923 and World War II [4]. The Rabies Prevention Law was put into effect in 1950 to stipulate registration and vaccination of domestic dogs, to capture and detain of stray dogs, and to implement quarantine for imported and exported dogs, resulting in eradication of rabies in Japan. In addition, few wild animal species, which can cause an epidemic of rabies inhabiting in Japan, and geographical advantages as an island country contribute to the successful eradication.

No incidence of indigenous human rabies had been reported for a long time since 1956. Only two cases were found in indigenous feline rabies (1957) and imported human rabies from Nepal (1970). In November 2006, two cases imported from the Philippines occurred one after another, as described above. It is apprehensive that imported rabies can occur in the future in consideration of increased number of overseas tourists due to growing globalization.

2. Epidemic Situations of Rabies in the World Including Developed Countries

WHO reports that rabies-induced mortality totals 55,000 persons a year [5], which is, however, considered to be an underestimated number. Outbreaks of rabies have