

Table 2. Difference in virulence between the Zr-599 and Liberia strains of monkeypox virus in non-human primates, as determined by the proposed scoring system for analysis of monkeypox severity

Abbreviations: UDL, under detection level; WBC, white blood cells.

Item	No. animals with score	Mean score in each group			
		Zr-599/SC	Zr-599/IN	Liberia/SC	Liberia/IN
Decrease in body mass (%)	<3, 0; 3-8, 1; 8-13, 2; >13, 3	2.3 ± 0.5	1.5 ± 0.7	2.3 ± 1.2	2.0 ± 0.0
Recovery signs in body mass	Positive, 0; negative, 3	2.3 ± 1.5	0	1.0 ± 1.7	0
Duration of decreased activity (days)	None, 0; 1-5, 1; 6-10, 2; >10, 3	2.3 ± 1.0	1.5 ± 0.7	2.0 ± 0.0	1.5 ± 0.7
Duration of decreased meal consumption (days)	None, 0; 1-5, 1; 6-10, 2; >10, 3	2.5 ± 0.6	2.0 ± 1.4	2.3 ± 0.6	1.0 ± 0.0
Fever >1 °C	Negative, 0; positive, 1	0.3 ± 0.5	0	0.3 ± 0.6	0
Drop in body temperature >1.5 °C	Negative, 0; positive, 3	2.3 ± 0.5	0	1.0 ± 1.7	0
Faecal appearance	Normal, 0; watery diarrhoea, 1; haemorrhagic diarrhoea, 3	1.8 ± 1.5	1.5 ± 2.1	0.7 ± 0.6	1.0 ± 0.0
Papulovesicular lesions (no.)	None, 0; 1-50, 1; 51-499, 2; >500, 3	2.3 ± 0.5	1.5 ± 0.7	2.0 ± 1.0	1.0 ± 0.0
Outcome	Non-fatal, 0; fatal, 6	4.5 ± 3.0	0	2.0 ± 3.5	0
Maximum virus genome level [\log_{10} (copies ml ⁻¹)]	UDL, 0; <5, 1; 5-7, 2; >7, 3	2.8 ± 0.5	2.5 ± 0.7	2.3 ± 0.6	1.5 ± 0.7
Virus genome level when sacrificed [\log_{10} (copies ml ⁻¹)]	UDL, 0; <5, 1; 5-7, 2; >7, 3	2.0 ± 1.4	1.0 ± 1.4	1.0 ± 1.0	0.5 ± 0.7
Peripheral WBC count <5000 µl ⁻¹	Positive, 0; negative, 1	0.5 ± 0.6	1.0 ± 0.0	1.0 ± 1.0	0.5 ± 0.7
Thrombocytopenia <20 000 µl ⁻¹	Positive, 0; negative, 1	0.8 ± 0.5	0.5 ± 0.7	0.3 ± 0.5	1.0 ± 0.0
Anaemia with decrease in haemoglobin level >1.5 g dl ⁻¹	Positive, 0; negative, 1	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
Maximum CRP level [mg dl ⁻¹]	<1, 0; 1-5, 1; 5-10, 2; >10, 3	2.8 ± 0.5	1.5 ± 0.7	2.3 ± 0.6	1.0 ± 1.4
Mean		30.0 ± 10.4	15.5 ± 9.2	20.7 ± 9.1	11.5 ± 3.5

in the Zr-599-infected monkeys than in the Liberia-infected monkeys.

The difference in the level and course of virus genome detection was consistent with the difference in the pathological findings. Zr-599 replicated more efficiently in the internal organs than did Liberia (Fig. 1; Table 1). The higher level of virus genome detected in the later phase of infection in the Zr-599/SC monkeys might be due to the more efficient replication of Zr-599, particularly in the generalized lymphoid and reticuloendothelial systems, skin, genito-urinary tract organs, respiratory organs and gastrointestinal organs, than that of Liberia in these organs. This feature of Zr-599 might lead to multi-organ failure with malfunctions of respiratory, gastrointestinal and genito-urinary tract organs, resulting in stronger virulence of Zr-599 than of Liberia in non-human primates.

It is believed that Congo Basin strains are more virulent than West African strains in humans, based on clinical studies (Breman *et al.*, 1980; Foster *et al.*, 1972; Ladnyj *et al.*, 1972). Recently, it was reported that a Congo Basin strain, Zr79, which was isolated from a fatal case of monkeypox in Zaire in 1979, was more virulent than the West African strain US03, which was isolated from a non-fatal case of monkeypox in the USA in 2003, using a ground squirrel model for monkeypox virus infection (Sbrana *et al.*, 2007). The clinical course and virological

and pathophysiological features of monkeypox virus infections in non-human primates obtained in this study were different from those reported in the ground squirrel model (Sbrana *et al.*, 2007; Tesh *et al.*, 2004). Monkeypox virus infections in non-human primates resemble human monkeypox in terms of pathophysiological profile, making the present study of particular value.

The genetic and molecular mechanism(s) underlying the differences in pathogenesis between Congo Basin and West African strains should be clarified through further studies. It was reported that *D10L*, *D14L*, *B10R*, *B14R* and *B19R* were possibly responsible genes, with *D14L*, an orthologue of vaccinia complement protein, as a leading candidate and with *D10L* and *B19R* as less likely candidates (Chen *et al.*, 2005). Although the data are not shown here, the *D14L* gene was confirmed to be absent in the Liberia strain, as in the case of an West African strain, SL-70 (Chen *et al.*, 2005).

In conclusion, it was demonstrated that Zr-599, a Congo Basin strain, was more virulent than Liberia, a West African strain. The difference in virulence might be due to the difference in the sites of virus replication resulting in organ dysfunction: Zr-599 replicated in skin, lymphoid and reticuloendothelial systems, genito-urinary tract organs, respiratory organs and gastrointestinal organs, whereas Liberia replicated only in skin, lymphoid and reticuloendothelial systems.

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Characterization of Monoclonal Antibodies to Junin Virus Nucleocapsid Protein and Application to the Diagnosis of Hemorrhagic Fever Caused by South American Arenaviruses[∇]

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Junin virus (JUNV), Machupo virus, Guanarito virus, Sabia virus, and Chapare virus are members of New World arenavirus clade B and are the etiological agents of viral hemorrhagic fevers that occur in South America. In this study, we produced three monoclonal antibodies (MAbs) to the recombinant nucleocapsid protein of JUNV, designated C6-9, C11-12, and E4-2. The specificity of these MAbs was examined by enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence assay, and an epitope-mapping method. Using these MAbs, we developed antigen (Ag) capture ELISA systems. We showed that by using MAb C6-9, JUNV Ag was specifically detected. On the other hand, by using MAb C11-12 or E-4-2, the Ags of all human pathogenic South American arenaviruses were detected. The combined use of these Ag capture ELISA systems in the present study may be useful for the diagnosis of acute-phase viral hemorrhagic fever due to infection by a South American arenavirus.

The South American arenaviruses Junin virus (JUNV), Machupo virus (MACV), Guanarito virus (GTOV), Sabia virus (SABV), and Chapare virus (CHPV) are members of New World arenavirus clade B. JUNV, MACV, GTOV, and SABV are the etiological agents of Argentine hemorrhagic fever (AHF), Bolivian hemorrhagic fever (BHF), Venezuelan hemorrhagic fever (VHF), and Brazilian hemorrhagic fever, respectively (4). CHPV was also recently shown to be associated with cases of hemorrhagic fever in Bolivia (5). AHF emerged in the 1950s, and since then, outbreaks have occurred annually without interruption (4). The mortality rate for AHF is estimated to be 15 to 30%, but early treatment with immune plasma reduces the rate to less than 1% (6). The region at risk has been progressively expanding into northern central Argentina, and almost 5 million people are currently considered to be at risk for AHF (6, 13). Phylogenetic analysis indicates that JUNV is more closely related to MACV than to SABV or CHPV, whereas SABV and CHPV are more closely related to each other than to other New World arenaviruses (5).

Arenaviruses are enveloped and contain a bisegmented RNA genome. The genome consists of two ambisense single-stranded RNA molecules, one designated L, which encodes the RNA-dependent RNA polymerase and a zinc-binding matrix protein, Z, and the other designated S, which encodes the major structural components of the virion, i.e., the nucleocap-

sid protein (NP) and the envelope glycoprotein precursor (15). The arenavirus NP is the most abundant protein among the viral structural proteins both in infected cells and in virions (2) and is commonly used as a target for detecting viral antigens (Ags) (20). Moreover, arenavirus NPs have been known to be the most conserved among the same virus species and, to some extent, among different arenavirus species (3, 8). Therefore, it seems likely that monoclonal antibodies (MAbs) raised against the NP of an arenavirus would also be useful for detecting other arenaviruses (20). Recently, an immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) was developed by using a recombinant NP (rNP) of JUNV, obtained from a recombinant baculovirus system, and was proposed to be useful for etiologic confirmation of AHF in seroepidemiological studies (20, 26). It is considered that an Ag capture ELISA using MAbs specific for viral Ags allows rapid diagnosis of the acute phase of viral hemorrhagic fever by detecting viral Ags in blood or tissue homogenates (20). In this study, we produced MAbs to the rNP of JUNV. These MAbs were characterized by ELISA, indirect immunofluorescence assay (IFA), and an epitope-mapping method. Ag capture ELISAs were developed by using these MAbs that are specific for JUNV and that are broadly applicable for the detection of human pathogenic New World arenaviruses.

MATERIALS AND METHODS

Cell culture. Hybridomas and their parental cell line, P3/Ag568, were maintained in RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), nonessential amino acids (Invitrogen), and antibiotics (streptomycin and penicillin G; Invitrogen). Hypoxanthine-aminopterin-thymidine supplement (Invitrogen) was added to the me-

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dium for selection of hybridomas, as recommended by the supplier. BTI-TN-5B1-4 (High Five; Invitrogen) insect cells were maintained in TC100 (Invitrogen) supplemented with 10% FBS, 2% tryptose phosphate broth (Difco, Detroit, MI), and kanamycin (Invitrogen). HeLa cells were maintained in minimal essential medium (Sigma-Aldrich, St. Louis, MO) supplemented with 5% FBS and antibiotics (streptomycin and penicillin G; Invitrogen).

Recombinant baculoviruses. The baculoviruses Ac-JUNV-NP and Ac-His-Lassa virus (LASV)-NP, expressing the JUNV and His-LASV rNPs, respectively, were generated as described previously (20).

The cDNAs of the MACV, GTOV, SABV, and CHPV NPs were obtained by chemical synthesis (Codon Devices, Cambridge, MA). The GenBank accession numbers of the nucleotide sequences of the MACV, GTOV, SABV, and CHPV NP genes are NC_005078, AF485258, NC_006317, and NC_010562, respectively. The cDNAs of the MACV, GTOV, SABV, and CHPV NPs were digested with BamHI and subcloned into the BamHI restriction site of pAcYM1 (14), and the resulting plasmids were designated pAcYM1-MACV-NP, pAcYM1-GTOV-NP, pAcYM1-SABV-NP, and pAcYM1-CHPV-NP, respectively. High Five cells were transfected with mixtures of linearized BacPAK6 DNA (Clontech, Mountain View, CA) and the recombinant transfer vector according to the manufacturer's instructions and the procedures described by Kitts and Possee (10), and recombinant baculoviruses were obtained from them. The baculoviruses expressing the MACV, GTOV, SABV, and CHPV rNPs were designated Ac-MACV-NP, Ac-GTOV-NP, Ac-SABV-NP, and Ac-CHPV-NP, respectively.

Expression and purification of rNPs. High Five cells infected with Ac-JUNV-NP, Ac-MACV-NP, Ac-GTOV-NP, Ac-SABV-NP, Ac-CHPV-NP, or Ac-His-LASV-NP were incubated at 26°C for 72 h. The cells were then washed twice with cold phosphate-buffered saline (PBS) solution. The High Five cells were lysed in PBS containing 1% NP-40 and 2 M urea. After the cell lysates were centrifuged at 15,000 × g for 10 min, the pellet fractions were collected and then solubilized in PBS containing 8 M urea. After the samples were centrifuged, the supernatant fractions were used as the purified Ags. The control Ag was produced from High Five cells infected with Ac-ΔP, which lacks the polyhedrin gene, in the same manner as for the negative control Ags. All Ags were aliquoted and kept at -80°C until use.

Establishment of MAbs. BALB/c mice were immunized three times with the purified JUNV rNP. Spleen cells were obtained 3 days after the last immunization and fused with P3/Ag568 cells by using polyethylene glycol (Invitrogen). The culture supernatants of the hybridoma cells were screened by ELISA with purified JUNV rNP as an Ag in the presence of 2 M urea. MAbs were purified from the culture supernatant by using a MABTrap GII antibody purification kit (GE Healthcare Bio-Sciences, Piscataway, NJ) according to the manufacturer's instructions. The concentration of each purified MAb was also determined by use of a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

Polyclonal antibodies. Polyclonal antibodies were induced in rabbits by immunization with the purified rNPs of JUNV, MACV, GTOV, SABV, and CHPV, respectively. Rabbit sera collected before immunization were used as controls.

IgG ELISA. The IgG ELISA was performed as previously described, except for Ag preparation (20–22). Briefly, ELISA plates (96 wells, Pro-Bind; Falcon; Becton Dickinson Labware, Franklin Lakes, NJ) were coated with the predetermined optimal quantity of purified JUNV, MACV, GTOV, SABV, CHPV, or His-LASV rNP (approximately 100 ng/well) at 4°C overnight. Then, each well of the plates was covered with 200 μl of PBS containing 5% skim milk and 0.05% Tween 20 (PBST-M), followed by incubation for 1 h for blocking at 37°C. The plates were washed three times with PBS containing 0.05% Tween 20 (PBST) and then inoculated with MAbs (100 μl/well), which were diluted 1:1,000 with PBST-M. After a 1-h incubation period, the plates were washed three times with PBST and then the plates were inoculated with goat anti-mouse IgG antibody labeled with horseradish peroxidase (HRP; 1:1,000 dilution; Zymed Laboratories, Inc., South San Francisco, CA). After a further 1-h incubation period, the plates were washed and 100 μl of ABTS [2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid)] solution (Roche Diagnostics, Mannheim, Germany) was added to each well. The plates were incubated for 30 min at room temperature, and the optical density at 405 nm (OD₄₀₅) was measured against a reference of 490 nm. The adjusted OD₄₀₅ value was calculated by subtracting the OD₄₀₅ value of the negative Ag-coated wells from that of the corresponding wells.

IFA. The full-length cDNA of the JUNV NP obtained from Ac-JUNV-NP, which possessed a BamHI restriction site at both extremities, was cloned into the BamHI site of the pKS336 vector (23), and the resulting plasmid was designated pKS-JUNV-NP. Also, the chemically synthesized full-length cDNAs of the MACV, GTOV, SABV, and CHPV NPs were cloned into the BamHI site of the pKS336 vector and the resulting plasmids were designated pKS-MACV-NP,

pKS-GTOV-NP, pKS-SABV-NP, and pKS-CHPV-NP, respectively. HeLa cells were then transfected with each of these expression plasmids by using a transfection reagent (FuGENE6; Roche Diagnostics) according to the manufacturer's instructions. The transfected cells were selected with 4 μg of blasticidin 5-hydrochloride/ml in culture medium. The HeLa cell clones were obtained by analyzing the expression of each rNP by IFA with rabbit serum raised against the JUNV, MACV, GTOV, SABV, or CHPV rNP, as previously described (20).

Ag capture ELISA. Purified MAb C6-9, C11-12, or E4-2 was used to coat microwell immunoplates (Falcon; Becton Dickinson Labware) at 100 ng/well in 100 μl of PBS at 4°C overnight, followed by blocking with PBST-M for 1 h at room temperature. After the plates were washed with PBST, 100 μl of samples containing serially diluted rNP of JUNV, MACV, GTOV, SABV, CHPV, or LASV was added and the plates were incubated for 1 h at 37°C. The plates were then washed with PBST, and 100 μl of rabbit polyclonal antibody raised against the rNP of JUNV diluted 1:500 with PBST-M was added to each well. After 1 h of incubation at 37°C, the plates were washed with PBST, and HRP-conjugated goat anti-rabbit IgG (Zymed, San Francisco, CA) was added. The plates were incubated for 1 h at room temperature. After another extensive washing with PBST, 100 μl of ABTS substrate solution (Roche Diagnostics) was added and the OD₄₀₅ was measured with a reference wavelength of 490 nm after 30 min of incubation at room temperature. As a negative control, the OD of control Ag-inoculated wells was measured. The adjusted OD₄₀₅ values were calculated by subtracting the OD₄₀₅ value of the negative control well from the corresponding OD₄₀₅ values. Means and standard deviations were calculated from the ODS of 12 negative control wells, and the cutoff value for the assay was defined as the mean plus 3 standard deviations.

Expression of truncated rNPs of JUNV. In order to determine the epitope on the JUNV rNP for the MAbs, a series of truncated JUNV rNPs were expressed as fusion proteins with glutathione S-transferase (GST). The DNA corresponding to each of the truncated NP fragments was amplified by PCR with specifically designed primer sets. The amplified DNA was subcloned into the BamHI and EcoRI cloning sites of plasmid pGEX-2T (Amersham Pharmacia Biotech, Buckinghamshire, England). The GST-tagged full-length rNP (GST-JUNV frNP) or truncated forms of the rNP (GST-JUNV trNPs) were expressed in *Escherichia coli* BL21 and then partially purified.

Western blotting. The MAbs were tested for reactivity to GST-JUNV frNP and a series of GST-JUNV trNPs by Western blotting as reported previously (9, 18, 24).

Mab epitope mapping. The epitopes for MAbs C6-9 and C11-12 were determined by epitope-blocking ELISA using synthetic peptides. The decapeptides were chemically synthesized by shifting one amino acid, with a consecutive overlap of nine amino acids to cover the JUNV NP (amino acids [aa] 5 to 26 for C6-9 and aa 543 to 564 for C11-12). ELISA plates were coated with purified JUNV rNP prepared by using a baculovirus expression system (approximately 100 ng/well) at 4°C overnight. Then, each well of the plates was inoculated with 200 μl of PBS-M, followed by incubation for 1 h for blocking. MAb C6-9 or C11-12 was mixed with each peptide (1 μg/well) and incubated for 1 h at 37°C, and then the mixture was added to each well of the plates. After a 1-h incubation period, the plates were washed three times with PBST, and then the plates were inoculated with goat anti-mouse IgG antibody labeled with HRP (1:1,000 dilution; Zymed). The following procedure was performed as described in the IgG ELISA section above.

For MAb E4-2, the epitope was determined by ELISA using GST-JUNV frNP and trNPs. ELISA plates were coated with purified GST-JUNV frNP or trNPs (approximately 100 ng/well) according to the method described in the IgG ELISA section above. MAb E4-2 or an anti-GST MAb was used for detection at a 1:2,000 or a 1:500 dilution, respectively. The adjusted OD₄₀₅ was calculated by dividing the OD₄₀₅ of MAb E4-2 by that of the anti-GST MAb from the corresponding wells.

RESULTS

Generation of MAbs. In order to obtain MAbs against the JUNV NP, BALB/c mice were immunized with the purified rNP of JUNV. The MAbs were purified and tested for reactivity to the rNP of JUNV by IgG ELISA. Three MAbs, designated MAb C6-9, MAb C11-12, and MAb E4-2, reacted with the rNP of JUNV by IgG ELISA even in the presence of 2 M urea.

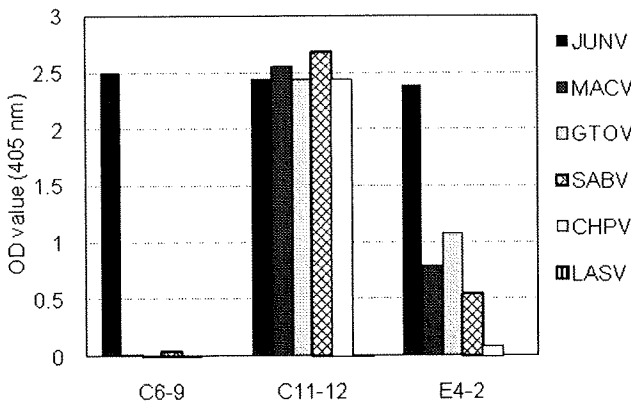


FIG. 1. Reactivity of each MAb with arenavirus rNP. Each purified rNP (100 ng/well) was used to coat microplates as described in the text, and the reactivities of each MAb to the rNPs of JUNV, MACV, GTOV, SABV, CHPV, and LASV were measured. The MABs are shown on the x axis. Results are expressed as the OD₄₀₅.

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

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.

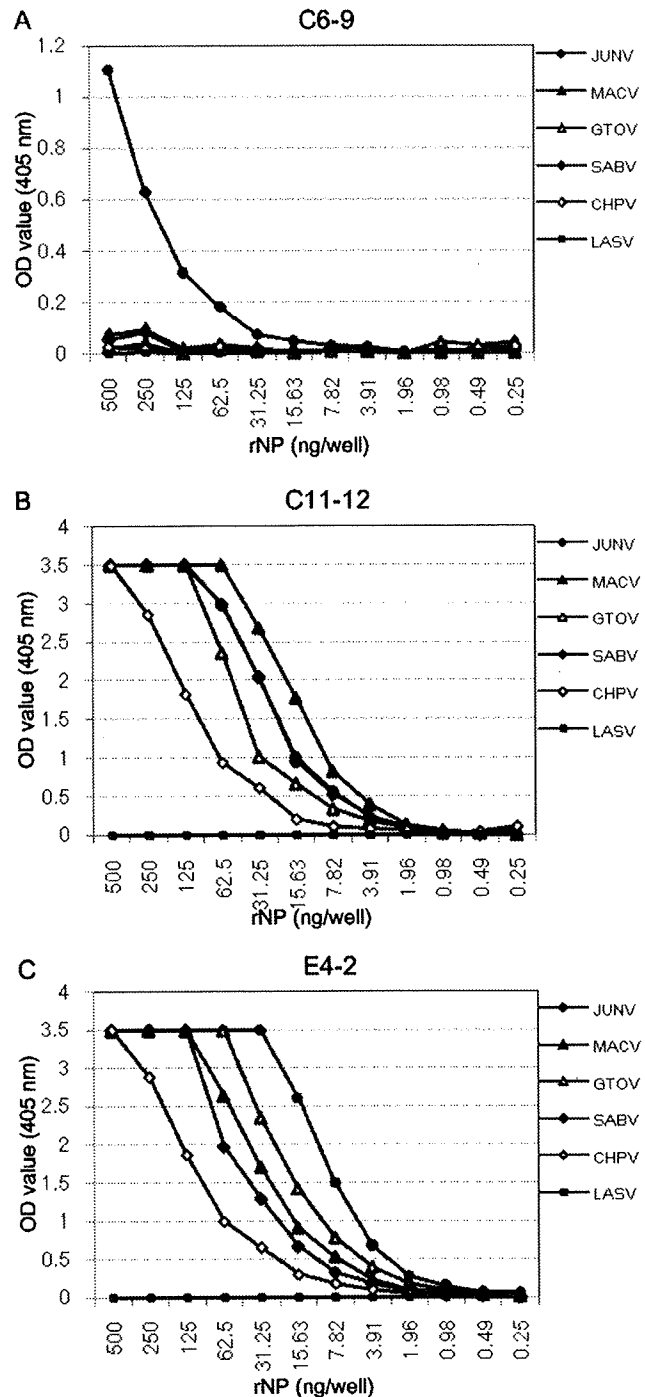


FIG. 2. Reactivity of each MAB in an Ag capture ELISA. Purified MABs C6-9 (A), C11-12 (B), and E4-2 (C) were used to coat microplates as described in the text, and their abilities to capture the rNPs of JUNV, MACV, GTOV, SABV, CHPV, and LASV were examined at various concentrations in the Ag capture format. Results are expressed as the OD₄₀₅.

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

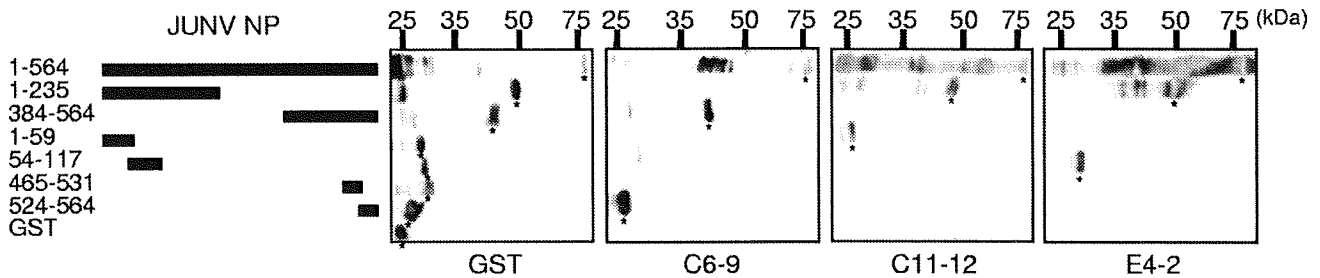


FIG. 3. Reactivities of MAbs C6-9, C11-12, and E4-2 with the GST-tagged JUNV NP by Western blotting. Schematic drawings of polypeptides of the JUNV NP and the amino acid positions of the polypeptides are shown on the left. The reactivities of MAbs and a control MAb against GST to these polypeptides by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

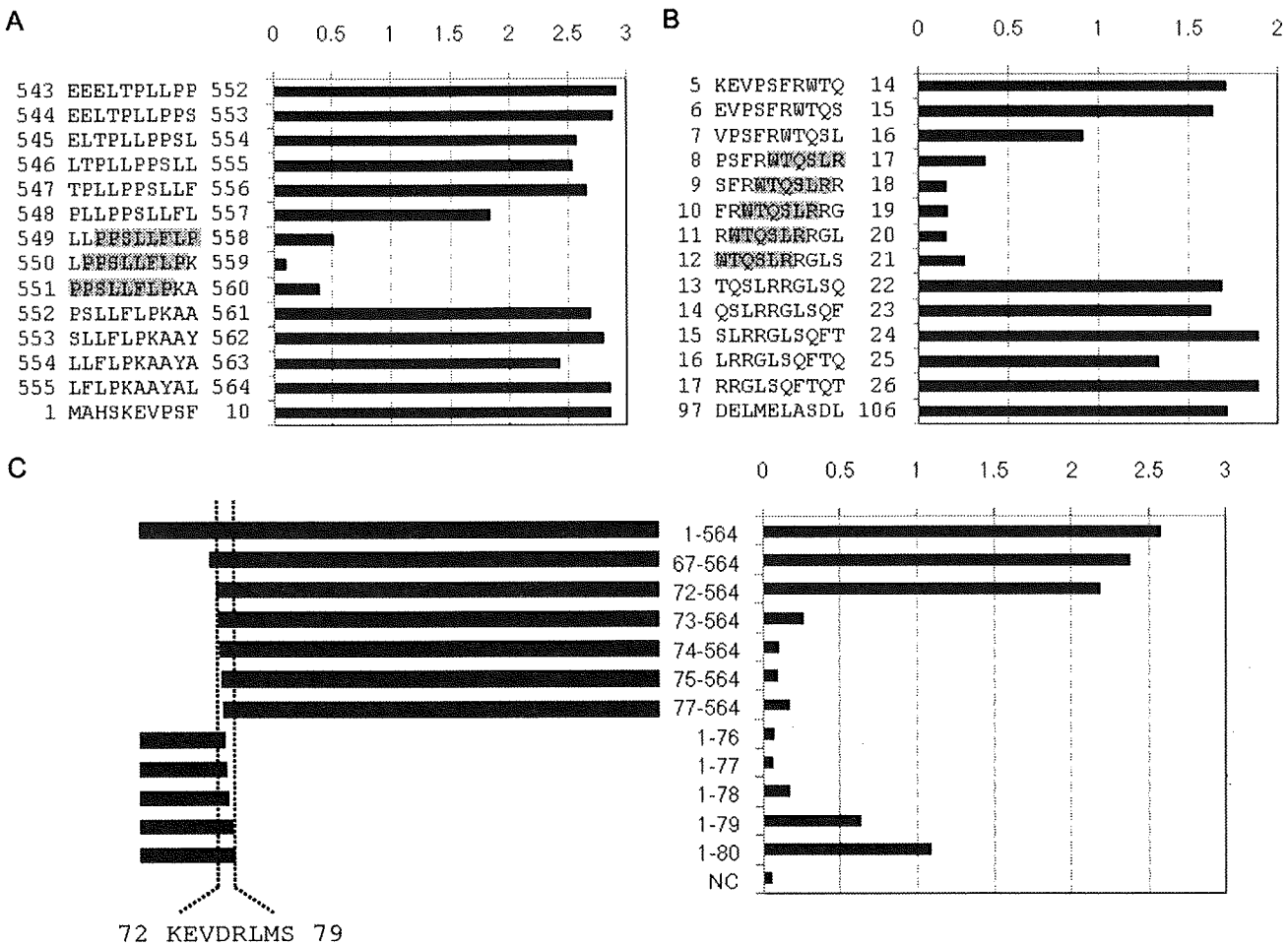


FIG. 4. Determination of the epitope on JUNV NP recognized by MAb C6-9 (A), MAb C11-12 (B), and MAb E4-2 (C). (A, B) The ability of synthetic decapeptides to block the reactivity of MAb to the JUNV rNP was examined by ELISA. The amino acid sequences and positions of synthetic peptides used in the assay 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 MAb C6-9 and MAb 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 MAb C11-12 and E4-2 (aa 1 to 80) was relatively conserved among the NPs of South American arenaviruses, Ag capture ELISAs using MAb 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.

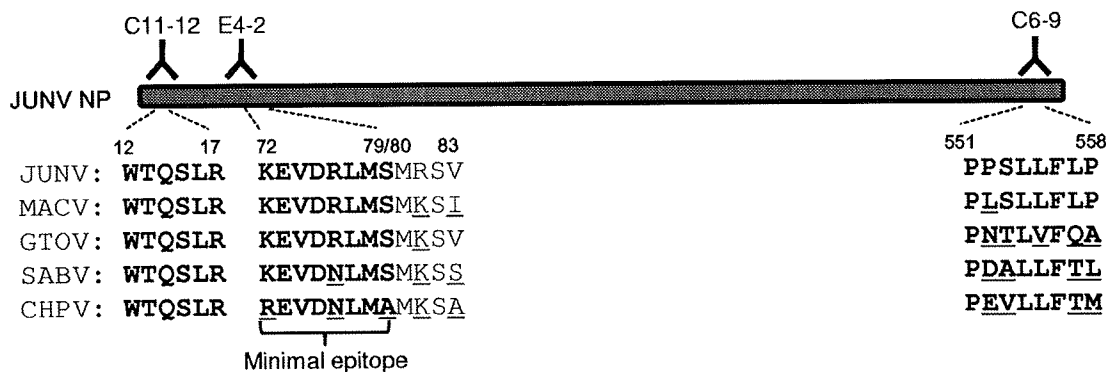


FIG. 5. Schematic representation of epitopes of the JUNV NP recognized by MABs. The amino acid sequences of the epitopes of the JUNV NP recognized by MABs C6-9, C11-12, and E4-2 are in boldface, and the amino acid positions are shown above the sequence. The amino acid sequences of the epitopes are compared 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.

ACKNOWLEDGMENTS

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Medical Conference via e-medicine

資料

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○ “Swine Flu an International Update”

〈参加機関〉

Royal North Shore Hospital

Bach Mai Hospital

Rinku General Medical Center

International Medical Center of Japan

〈開催日時〉 2009年8月25日

○ “国際的感染症診療ネットワーク会議（一類感染症を含む）”

〈参加機関〉

国立国際医療センター

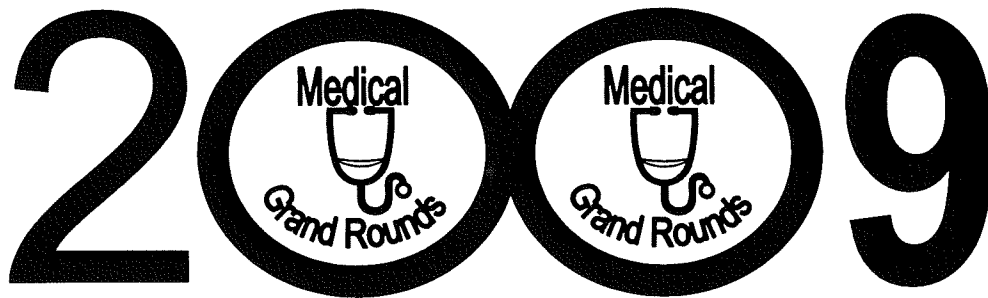
りんくう総合医療センター市立泉佐野病院

成田赤十字病院

成田空港検疫所

東京検疫所 東京空港検疫所支所

〈開催日時〉 2010年3月10日



Medical Grand Rounds

“Swine Flu – an International Update”

**Medical Conference via e-medicine
Sydney – Hanoi – Osaka - Tokyo**

Tuesday 25 August

Royal North Shore Hospital , the University of Sydney, Sydney, Australia

Bach Mai Hospital, Hanoi, Vietnam

Rinku General Medical Center, Osaka, Japan

International Medical Center of Japan, Tokyo, Japan

Grand Rounds:Swine Flu Symposium

August 25th 2009.

Royal North Shore Hospital

Sydney/Hanoi/Tokyo/Osaka

Chairman:Prof.C.Dennis

Program

1. Welcome Prof.Jonathan Morris(Hoc Mai Foundation)&Chairman's introduction

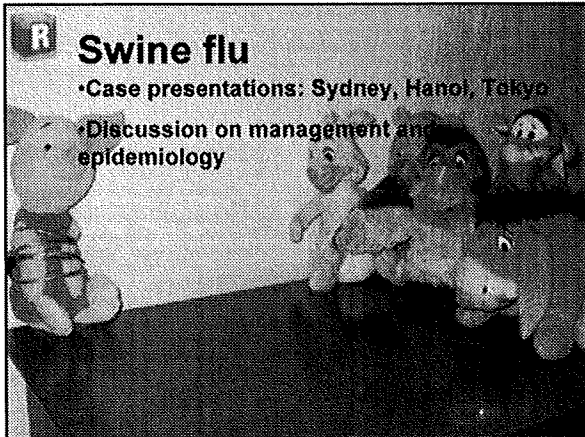
2. Case 1(Sydney)
Dr.Melanie Figtree(RNSH)
Question 1:Dr.Robyn Hardiman
Discussion

3. Case 2(Hanoi)
Dr.So Duy Cuong(Bach Mai Hospital)
Question 2:Prof.Ngo Quy Chau
Discussion

4. Case 3
Dr.Jin Takasaki(IMCJ)
Question 3:Dr.Koichiro Kudo
Discussion

5. Questions 4&5
General discussion

6. Finish



Swine flu

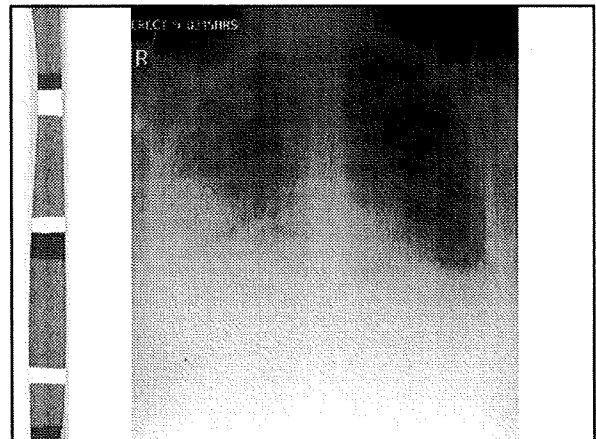
- Case presentations: Sydney, Hanoi, Tokyo
- Discussion on management and epidemiology

Ms X: Presentation

- 21 year old woman 34 weeks pregnant
- Extreme respiratory distress
- 3 week history of feeling generally unwell, sore throat, fever, myalgias, increasing respiratory distress, cough productive sputum
- Background: body mass index 25 prior to pregnancy
- Sister had suffered similar illness
- Works as a babysitter. No recent travel.
- Non smoker

Assessment

- Respiratory
 - Speaking in words
 - Respiratory rate ~30 breaths per minute
 - Room air SaO₂ 85%
 - 15L NRBM SaO₂ 91%
 - Bilateral crackles
 - Arterial blood gas on 40% O₂
 - pH 7.44
 - pO₂ 61
 - pCO₂ 26
 - HC0₃ 13
 - BE -6
- Cardiovascular
 - Tachycardia 200 beats per minute
 - Diaphoretic
 - Dehydrated
- WCC 8.8 (lymph 0.7)
- CRP 146
- ALT 49 AST 99



Management

- Transferred to ICU at tertiary referral hospital requiring intubation/ventilation
 - Commenced oseltamivir 75mg bd 5 days
 - Antibiotic cover tazocin
 - Developed rash changed to Vancomycin/meropenem
- Emergency Caesarian in ICU
 - Baby girl 34 weeks gestation 2.4kg
 - Apgar scores 1/5/7
 - Hyaline membrane disease requiring mechanical ventilation 13 hours followed by CPAP for 12 hours

Ms X: Microbiology

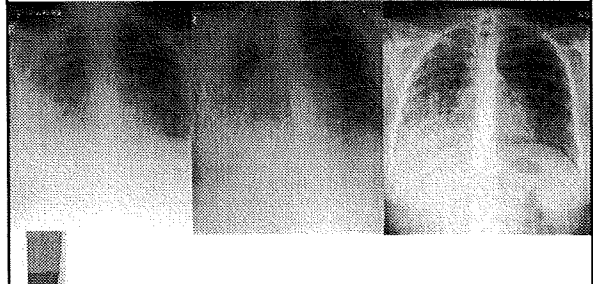
- Day 1: Nasal swab PCR
 - Flu A negative
 - Novel H1N1 negative
- Day 1: Throat swab PCR
 - Novel H1N1 negative
- Day 2: Tracheal aspirate
 - Immunofluorescence Influenza A negative
 - PCR positive novel H1N1

Further Management

- Mechanical ventilation 17 days
- Intensive care 19 days
- High ventilation requirements, slow wean
- Extracorporeal Membrane Oxygenation (ECMO) considered

Radiology


Admission	Day 10	Day 20
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Outcome

- Complications
 - Premature delivery of baby
 - Deep venous thrombosis proximal subclavian vein
- Mum and baby discharged Day 24/Day 23

Overview situation of H1N1 flu in Vietnam



Bach Mai hospital

25 Aug 2009


Situation of H1N1 flu in Vietnam (As of 22 Aug 2009)

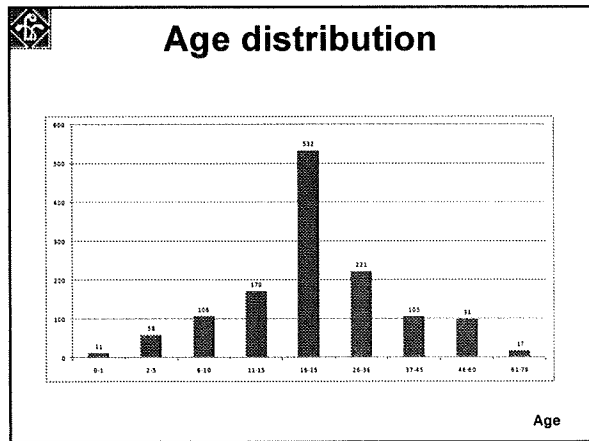
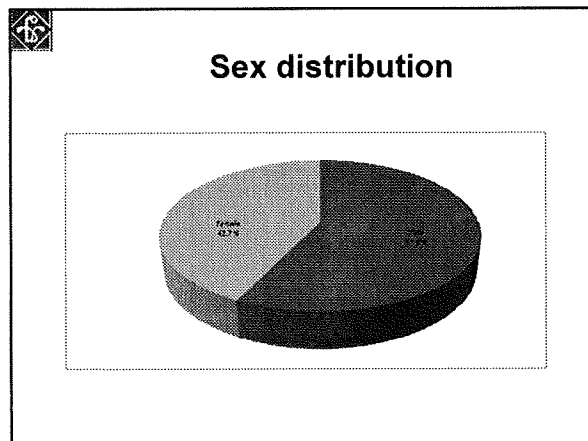
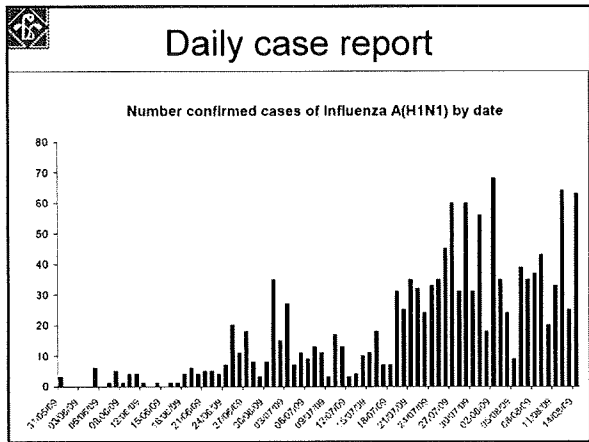
- New cases detected: 49 (37 South, 2 North, 8 Central and 2 Central highland).
- As of 17h00 of 22 Aug 2009, number of confirmed cases reported: **1,896**, deaths: **02**.
- Number of patients who were recovered and discharged: **1,193**.
- Number of patients are still isolated, monitored and hospitalized: **701**

Source: MoH - Vietnam
<http://www.moh.gov.vn/homeby/vn/portal/InfoDetail.jsp?area=58&cat=1456&ID=8011>

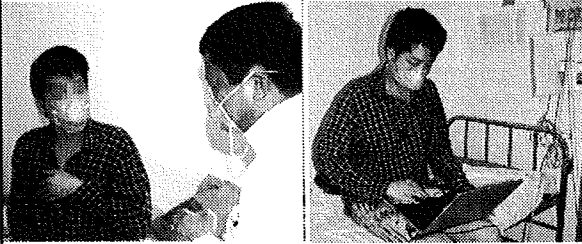
Province distribution of H1N1 flu in Vietnam

- 42 provinces:
 - 4 provinces with cases as in focal community.
 - 38 provinces with individual cases.





**The first case of H1N1 flu in Vietnam
(30 May 2009)**



- Male, 23 y/o
- Oversea Vietnamese
- Flew from Chicago to HCMC (26 May 2009)
- Discharged: 6 June 2009

2 death cases in Vietnam

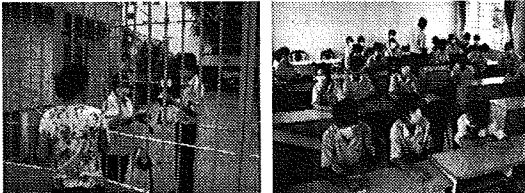
1st case:

- Female, 29 y/o, living in Nha Trang, Khanh Hoa province.
- Admitted to hospital: 30 Jul, 2009 with fever, coughing,...
- Ventilator was required.
- Died 3 Aug 2009

2nd case:


- Female, 52 y/o
- Living in District 10, HCMC.
- Admitted to hospital: 06 Aug, 2009 with fever, coughing, shortness of breath,...
- Died 10 Aug 2009

**The 1st focal outbreak in Ngo Thi Nham
high school – HCMC
(13 July 2009)**



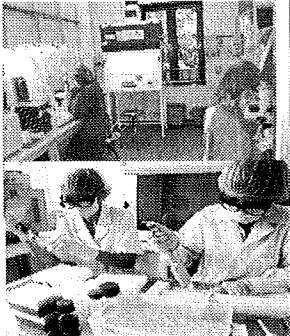
- 22 cases (3 teachers, 19 students).
- Contacted with a relative flown from the US.

Laboratory diagnostics

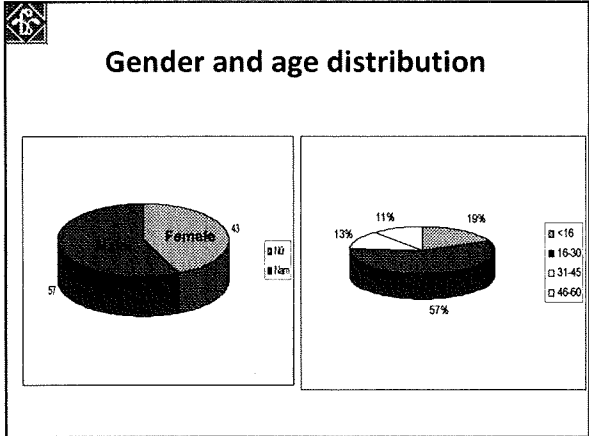


**Diagnostic laboratory for H1N1 flu in
Vietnam**

- Lab P3 (WHO standard) at:
 - National Institute for Hygiene and Epidemiology.
 - National Institute for Infectious and Tropical Diseases.
 - Pasteur Institute in Nha Trang.
 - Pasteur Institute in HCMC.
- Result available after 48 hours.



**Characteristics of 100 cases H1N1 attended National
Institute for Infectious and Tropical Diseases
(NIITD)**



- ### Clinical pictures
- Similar to seasonal flu:
 - Fever 100%, dry cough 50.0%, fatigue 42.3%, sore throat 30.8%, chill 7.7%, running nose 26.9%.
 - No respiratory failure.
 - Lab tests:
 - CXR normal.
 - Blood gas normal.
 - CK, CKMB, ALT, AST normal.
 - 28% of patients had presence of virus in the samples after 10 days of illness.

Prevention responses to the epidemic

THÔNG BÁO
Về tình hình dịch cúm A(H1N1)

Cục Y tế dự phòng và Môi trường, Bộ Y tế thông báo tình hình dịch cúm A(H1N1) đến 17h00 ngày 22/8/2009 như sau:

1. Tình hình dịch trên thế giới:

Theo thông báo của Tổ chức Y tế thế giới (WHO), đến ngày 20/8/2009, toàn thế giới đã ghi nhận 227.915 trường hợp dương tính với cúm A(H1N1) tại 182 quốc gia/vùng lãnh thổ, trong đó có 2.146 trường hợp tử vong.

Tại khu vực Đông Á và Đông Nam Á, tình hình dịch tiếp tục diễn biến phức tạp. Nhật Bản đã ghi nhận 03 trường hợp tử vong do cúm A(H1N1), với tình case khoảng 60.000 người đã nhiễm bệnh, Hàn Quốc (từ vùng 02), Philippines (từ vùng 06), Singapore (từ vùng 11), Brunei (từ vùng 01), Malaysia (từ vùng 67), Lào (từ vùng 03), Indonesia (từ vùng 03), Thái Lan (từ vùng 11).

2. Tình hình dịch tại Việt Nam:

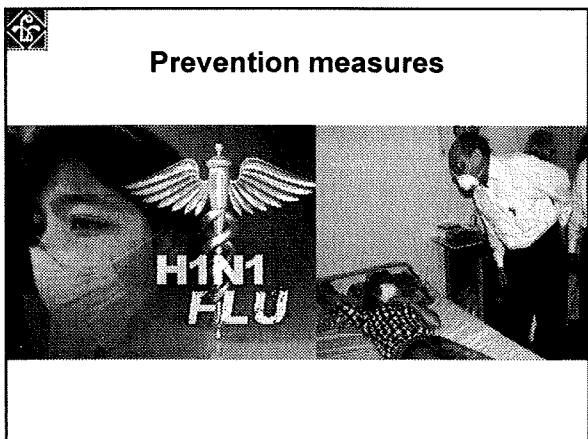
BỆNH VIỆN BẠCH MAI

Hướng dẫn chẩn đoán, điều trị và phòng lây nhiễm cúm lợn A (H1N1) ở người

QUYẾT ĐỊNH
Về việc ban hành "Hướng dẫn chẩn đoán, điều trị và phòng lây nhiễm cúm lợn A (H1N1) ở người"

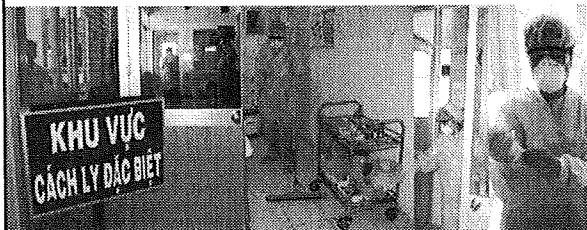
BỘ TƯỞNG BỘ Y TẾ

Căn cứ Nghị định số 189/2007/NĐ-CP ngày 27 tháng 12 năm 2007

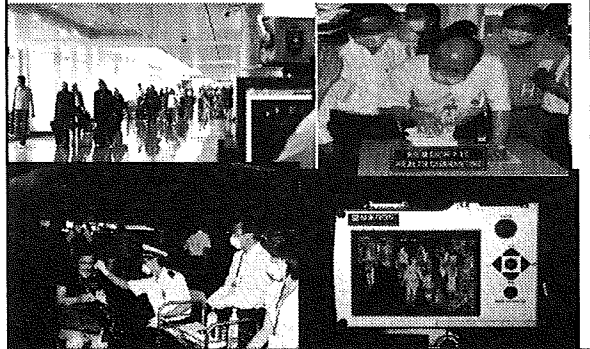




Isolation rooms in hospital



Passengers are quarantined for H1N1 flu at the airport



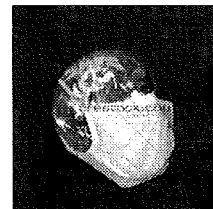
"Mask wearing" in the office and public



Hand washing and cleaning the environment



Thank you



Case Study from International Medical Center of Japan

Jin Takasaki, M.D.
Disease Control and Prevention Center
International Medical Center of Japan
Tokyo, Japan

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Case 1

Mother 48 y.o. F. Miami resident, US citizen
(two sons 16 y.o. M, 9 y.o. M)

C.C. (mother) Fever, Rhinorrhea, Cough, Chest pain

P.H. (mother) Thrombocytopenia

P.I.

2009/6/7 Family arrived Japan

6/8 Son (16 y.o.) fever-up to 39°C of acute
onset
with diarrhea

6/9 Mother and son (9 y.o.) got high fever (39°C)
rhinorrhea, cough.

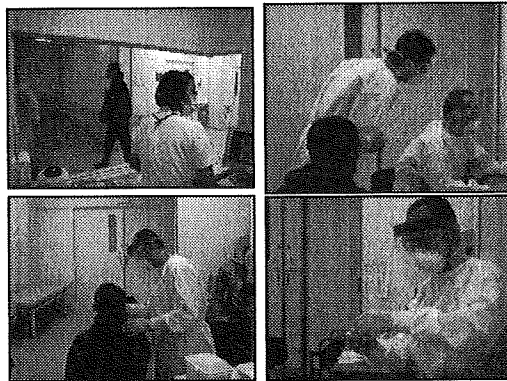
Influenza rapid diagnosis kit positive with nasal swab at A hospital

6/10 Confirmed S-OIV H1, positive

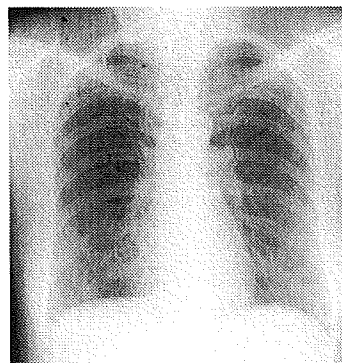
Administered in IMCJ

2

Fever Clinic in IMCJ



Chest X-ray on admission on June 10th



➢ Silhouette out sign in the right side of cardiac shadow

CRP 15.8 mg/dL

Chest CT on admission

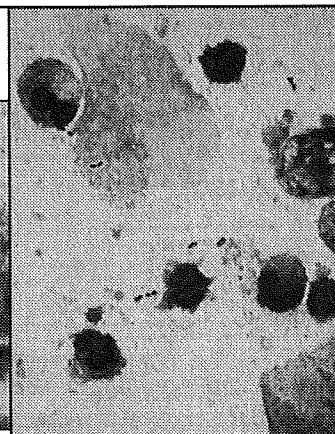


➢ Thickening bronchial wall of Rt middle lobe

➢ Infiltrative shadows in Rt middle lobe
➢ Bronchiectatic changes

➢ Infiltration and atelectasis of Rt middle lobe

Sputum gram stain
• gram positive.
• streptococcus pneumoniae



Summary

Co-infection or secondary infection of streptococci pneumoniae in RML with S-OIV.

Rx.)

Oseltamivir 150mg/day 2 × 5 days

AMPC 2. 2.5g/day 3 ×