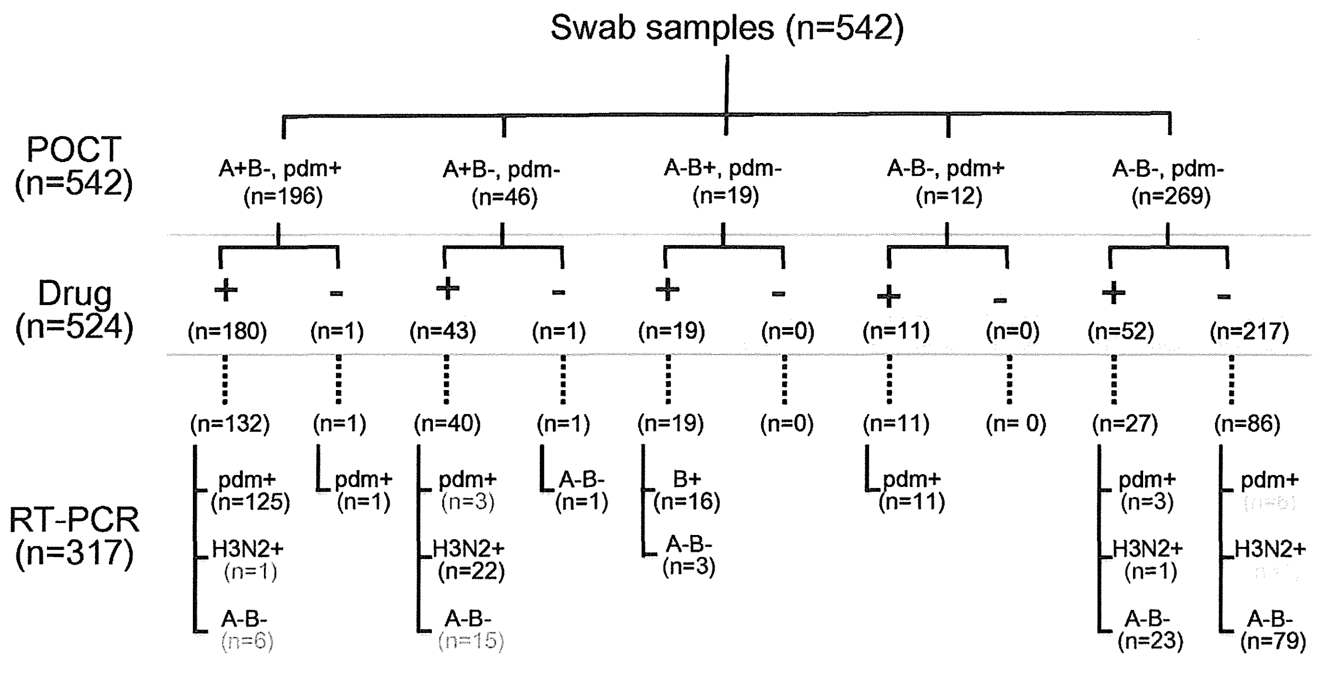

Figure 3. Summarized results for the sensitivity and specificity of the SEA-IC and PDM-IC test kits. First, a total of 542 swab samples were classified into five groups according to the results of POCT. Of these, anti-viral drug prescription information was available for 524 samples. These 524 samples were further classified into two groups: "+" for patients with anti-viral drug prescriptions and "−" for patients without. RT-PCR analysis was performed for 317 of the 524 samples. Numbers in green indicate false-positive IC test kit cases upon RT-PCR analysis, where the clinicians prescribed drugs according to the results of the IC kits. Numbers in blue indicate false-positive IC kit cases, but drugs were not prescribed. Numbers in red indicate negative IC kit cases that were confirmed by RT-PCR, where the clinicians prescribed drugs. Numbers in purple indicate false-negative IC kit cases upon RT-PCR analysis, where the clinicians prescribed drugs. Numbers in light blue indicate false-negative IC kit cases, where drugs were not prescribed although these cases tested positive by RT-PCR. Numbers in black indicate correctly diagnosed cases according to the results of POCT. Numbers in orange indicate that the RT-PCR analysis found detection errors in the IC kit results regarding the detection of H1N1pdm to H3N2 or H3N2 to H1N1pdm.
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positive) might be explained by the lower sensitivity of the IC kit compared with RT-PCR. On the other hand, there were several IC kit-derived false-positives (IC kit-positive, but RT-PCR-negative). One possible explanation for their occurrence might be the fact that viral antigens might persist even after virus particles had been removed from body. Alternatively, the monoclonal antibody employed by the kit might react with non-specific antigens derived from the host or from other pathogens. When the samples that gave inconsistent IC kit versus RT-PCR were examined by VI, several cases were observed that tested positive by VI, but negative by RT-PCR, especially for H1N1pdm. Furthermore, several additional cases tested negative by VI, but positive by RT-PCR, especially in H3N2. This indicates the possible difficulty of PCR amplification of target H1N1pdm DNA in the former case and of VI of H3N2 in the latter case. Regardless of the cause, further improvements in IC kits are required because false-negative and false-positive results lead to misdiagnosis upon POCT.

A total of 305 of 542 patients were treated with anti-viral drugs at 13 clinics in the present study. When the RT-PCR results were examined for 229 of these 305 drug-subscribed patients, we found that 23 individuals were given drugs even though they tested negative for the influenza virus by both IC test kits and RT-PCR. The drugs were dispensed because of patient responses to questionnaires regarding typical clinical symptoms of influenza, influenza infection in close relationships, friends, and/or colleagues, or an affected individual's desire to receive anti-viral drugs within the first day of fever occurrence, typically at a clinic that is open over the weekend. The latter case is thought by the conditions for influenza virus detection in such febrile patients might simply be too early, leading to false-negative results upon POCT.

Soon after the emergence of H1N1pdm, the virus was thought to be sensitive to oseltamivir (Tamiflu); however, several recent papers report an increasing incidence of drug-resistant H1N1pdm strains [5–8]. In 2004, an oseltamivir-resistant H3N2 strain was frequently detected in infants who were treated with the drug in Japan. This indicates a correlation between drug dosage and the manifestation of resistant strains [33]. Therefore, the use of an IC test kit with high sensitivity and specificity for H1N1pdm, together with a physician preference for IC test kit data instead of clinical influenza-like symptoms and patient responses to questionnaires, are strongly recommended prior to the prescription of anti-influenza drugs. Moreover, special attention must be paied to the fact that anti-viral drugs (e.g., oseltamivir) are used more heavily in Japan than in any other country in the world for the treatment and even prophylaxis of influenza. Although such drug use appears to have significantly reduced lethality due to H1N1pdm infections in Japan compared with other countries [16,17], the extensive use of anti-viral medication increases the risk of resultant drug-resistant influenza virus variants. The current study revealed that, since the appearance of H1N1pdm in 2009, anti-viral prescription practices of clinics in Japan are not always in accordance with the data afforded by IC kits. Therefore, the information provided by this study regarding the high reliability of concomitant use of SEA-IC and PDM-IC kits for the rapid diagnosis of influenza may lead to

reduced prescription of anti-viral drugs, as well as diminished emergence of drug-resistant virus variants.

The future global status of influenza viruses, including the evolution of H1N1pdm and/or the appearance of new pandemic strains, is largely unknown. Zhu et al. reported a new reassortant virus in swine in 2010 [34], and the Centers for Disease Control and Prevention reported a new case of swine-origin influenza A virus H3N2 in 2011 [35]. Thus, future surveillance of influenza viruses, followed by the rapid development of new IC kits for the diagnosis of such newly emerged viruses, is extremely important to human welfare. Because the PDM-IC test kit introduced in this study does not require any specialized equipment, it can be use both at the bedside and for field surveillance. Hence the PDM-IC kit and related kits are anticipated to be important tools for the identification and control of influenza viruses.

Supporting Information

Table S1 Duration of days after disease onset categorized by each age group.
(DOCX)

Table S2 Antiviral drug prescription information from 13 clinics for influenza virus-positive cases confirmed by RT-PCR.
(DOCX)

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Author Contributions

Conceived and designed the experiments: TS RKK MT MK KI. Performed the experiments: TS RKK. Analyzed the data: TS RKK MK. Contributed reagents/materials/analysis tools: MT TH SI TM KS DF MN EN AK YM MO YU TF. Wrote the paper: TS SK KI. Edited the manuscript: RKK SK MK YK. Provided oversight, support, and recruitment of study participants, as well as sample management: MT MK.

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Biologics: Targets and Therapy

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ORIGINAL RESEARCH

Limited cross-reactivity of mouse monoclonal antibodies against Dengue virus capsid protein among four serotypes

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Background: Dengue illness is one of the important mosquito-borne viral diseases in tropical and subtropical regions. Four serotypes of dengue virus (DENV-1, DENV-2, DENV-3, and DENV-4) are classified in the Flavivirus genus of the family *Flaviviridae*. We prepared monoclonal antibodies against DENV capsid protein from mice immunized with DENV-2 and determined the cross-reactivity with each serotype of DENV and Japanese encephalitis virus.
Methods and results: To clarify the relationship between the cross-reactivity of monoclonal antibodies and the diversity of these viruses, we examined the situations of flaviviruses by analyses of phylogenetic trees. Among a total of 60 prepared monoclonal antibodies specific for DENV, five monoclonal antibodies stained the nuclei of infected cells and were found to be specific to the capsid protein. Three were specific to DENV-2, while the other two were cross-reactive with DENV-2 and DENV-4. No monoclonal antibodies were cross-reactive with all four serotypes. Phylogenetic analysis of DENV amino acid sequences of the capsid protein revealed that DENV-2 and DENV-4 were clustered in the same branch, while DENV-1 and DENV-3 were clustered in the other branch. However, these classifications of the capsid protein were different from those of the envelope and nonstructural 1 proteins. Phylogenetic distances between the four serotypes of DENV were as different as those of other flaviviruses, such as Japanese encephalitis virus and West Nile virus. Large variations in the DENV serotypes were comparable with the differences between species of flavivirus. Furthermore, the diversity of flavivirus capsid protein was much greater than that of envelope and nonstructural 1 proteins.
Conclusion: In this study, we produced specific monoclonal antibodies that can be used to detect DENV-2 capsid protein, but not a cross-reactive one with all serotypes of DENV capsid protein. The high diversity of the DENV capsid protein sequence by phylogenetic analysis supported the low cross-reactivity of monoclonal antibodies against DENV capsid protein.
Keywords: Dengue virus, capsid protein, monoclonal antibody, cross-reactivity

Introduction

Dengue illness, such as dengue fever and dengue hemorrhagic fever/dengue shock syndrome, is one of the important mosquito-borne viral diseases in tropical and subtropical regions.[1] Four serotypes of dengue virus (DENV-1 to DENV-4) are classified in the genus Flavivirus of the family *Flaviviridae*. This also includes Japanese encephalitis virus, West Nile virus, and yellow fever virus.[2]

The 11 kb DENV genome is translated into a single polyprotein, which is subsequently processed by proteases into structural and nonstructural proteins. Three structural proteins, i.e. capsid, premembrane/membrane, and envelope, make up the virus particle.[3] The DENV capsid protein is a relatively small, highly positively

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charged 12 kDa protein and an essential factor during virion assembly.[4,5] Interestingly, DENV capsid protein is found in the nucleus and nucleoli of infected cells as early as 6 hours after infection, well before formation of infectious virus.[6,7] Previous reports have suggested nucleolar accumulation of DENV capsid protein may result from its interaction with RNA or nucleoli proteins related to regulation of ribosome synthesis, mRNA processing, and DNA replication.[5,8,9] This nuclear localization of capsid protein appears to be conserved among flaviviruses, including West Nile virus, Japanese encephalitis virus, Kunjin virus, and hepatitis C virus.[10–13]

In general, antibodies against the capsid proteins of viral particles, such as human immunodeficiency virus (HIV) Gag protein and influenza virus nucleoprotein, have been used in antigen-capture diagnosis kits[14,15] because of their wide range of cross-reactivity, even with other subtypes, compared with that of the envelope proteins.[16,17] Similar approaches using antigen-capture diagnostic or enzyme-linked immunoassay kits have been used for other viruses, such as Rift Valley fever virus,[18] hepatitis B virus,[19] hepatitis C virus,[20] and Ebola virus.[21] Such viral structural capsid proteins are located inside, not on the surface, of viral particles and the amount of internal protein exists at a higher concentration than that of surface protein. Therefore, use of monoclonal antibodies against the internal structural protein could have advantages for developing rapid diagnostic kits. In addition, expression of DENV capsid protein has been detected at a very early stage in infected mammalian cells.[33] Thus, if antibodies against DENV capsid protein were also widely cross-reactive among all four serotypes of DENV, like those against HIV Gag protein and influenza virus nucleoprotein, monoclonal antibodies recognizing common antigenic regions on DENV capsid proteins for all four DENV serotypes could be highly useful for developing a rapid diagnostic test kit widely cross-reactive with all four serotypes in the acute phase. However, monoclonal antibodies to the capsid protein has not been used for development of a diagnostic kit to detect this viral infection, and monoclonal antibodies to DENV nonstructural 1 protein have been used instead for developing diagnostic kits.[22,23]

In this study, we attempted to produce monoclonal antibodies against DENV capsid protein that could be cross-reactive with all four serotypes of DENV. By immunizing BALB/c mice with DENV-2, we obtained a total of five hybridoma clones producing specific antibodies against DENV capsid protein. We hypothesized that a monoclonal antibody against DENV capsid protein would recognize common antigenic regions on the capsid proteins of all four DENV serotypes and be very useful for developing a diagnostic test kit widely cross-reactive with all four serotypes. The purpose of this study was to clarify the relationship between the cross-reactivity of monoclonal antibodies and the diversity of these viruses, and we examined the situation of flaviviruses by analysis of phylogenetic trees.

Materials and methods

Cells and viruses

Vero cells (from an African green monkey kidney epithelial cell line) and B-7 cells (from a BALB/c mouse cell line)[24] were maintained in Eagle's Minimum Essential Medium (MEM, HyClone Laboratories Inc, Logan, UT) supplemented with 10% fetal calf serum (HyClone Laboratories Inc). PAI cells (from a mouse myeloma cell line) were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640, HyClone Laboratories Inc) medium supplemented with 10% fetal calf serum. All cell lines were cultured at 37°C in a 5% CO_2 incubator. C6/36 cells (from an *Aedes albopictus* cell line) were grown in Leibovitz-15 medium (HyClone Laboratories, Inc) supplemented with 0.3% tryptose phosphate broth and 10% fetal calf serum at 28°C.

Viral stocks of DENV-1 (Mochizuki strain), DENV-2 (New Guinea C strain), DENV-3 (H87 strain), and DENV-4 (H241 strain) were prepared as culture fluids from C6/36 cells infected with the individual serotypes and cultured for 7–9 days. In addition, the Nakayama strain of Japanese encephalitis virus was also similarly cultured in C6/36, and the culture fluid was used as a virus stock. Infectivity titers of these viruses were determined by the number of focus-forming units, as described previously.[25]

Mouse immunization and monoclonal antibody preparation

For preparation of DENV-2 antigens, we used two types of antigens, ie, B7-cells infected with DENV-2 and 50% brain homogenate with phosphate-buffered solution (–) from suckling BALB/c mice that were injected with DENV-2. B7-cells infected with DENV-2, at a multiplicity of infection of 0.1 and cultured for 2 days, were harvested by scraping and precipitated by centrifugation at 1000 rpm for 5 minutes. Brain homogenate was prepared by intracerebral injection of DENV-2 in suckling mice. As soon as the injected mice showed symptoms, their brains were collected and frozen at –80°C until use. Antigens were kept at –80°C until use for immunization of mice. Three 4-week-old female BALB/c mice (National Laboratory Animal Center, Mahidol University, Bangkok, Thailand) were immunized with 2.5×10^6 infected cells or 300 μL of homogenized brain

mixed with complete Freund's adjuvant (Sigma-Aldrich, Saint Louis, MO), as described previously.[26] Each mouse was injected intraperitoneally with 300 μL of mixed antigen. Immunized mice were intraperitoneally boost-immunized 3–4 times with similarly prepared antigens without adjuvant. This study was approved by the Faculty of Tropical Medicine Animal Care and Use Committee, Mahidol University, Bangkok, Thailand (FTM-ACUC 2011/003). Three days after the final booster immunization, splenocytes were prepared and subjected to fusion with PAI cells using polyethylene glycol 1500 (Roche Diagnostic Corporation, Basel, Switzerland). Fused cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 15% fetal calf serum and hypoxanthine-aminopterin-thymidine (Gibco, Grand Island, NY). Monoclonal antibodies produced from hybridomas were screened with DENV-2-infected Vero cells by an immunofluorescence assay. Hybridomas were cloned twice by limiting dilutions using 96-well microplates.

Expression of DENV-2 capsid protein

The DENV-2 capsid protein expression plasmid, pCAGGS-PM2 FLAG-DEN2 core 100-HA, was kindly provided by Y Matsuura at the Research Institute for Microbial Diseases of Osaka University, Suita, Osaka, Japan. Expression of this plasmid was confirmed by Western blotting and immunofluorescence assays using an anti-Flag M2 monoclonal antibody (Sigma-Aldrich). The plasmid vector was transfected by Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for Vero cells.

Immunofluorescence assay

Vero cells were seeded into 96-well plates for preparation of DENV antigens in infected cells and DENV-2 capsid protein in transfected cells. After incubation for 16–24 hours, they were infected with each serotype of DENV or transfected with pCAGGS-PM2 FLAG-DEN2 core 100-HA plasmid. Two days after infection or transfection, the cells were fixed with 4% paraformaldehyde in phosphate-buffered solution for 30 minutes at room temperature. Vero cells infected with Japanese encephalitis virus were also similarly prepared. The fixed cells were permeabilized with 1% Triton X-100 in phosphate-buffered solution for 5 minutes at room temperature, and then incubated with hybridoma culture fluid for one hour. They were then washed three times with phosphate-buffered solution and further treated with Alexa Fluor® 488 goat anti-mouse IgG antibody (Invitrogen) at a dilution of 1:500 for 45 minutes. Finally, they were washed three times with phosphate-buffered solution prior to observation by fluorescence microscopy (IX71, Olympus, Tokyo, Japan).

Western blotting assay

DENV-infected Vero cells were dissolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer with beta-mercaptoethanol and heated at 100°C for 5 minutes. The samples were separated in 12% SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane (Millipore Corporation, Bedford, MA). The membrane was incubated for 12 hours with antibody produced by the hybridoma clones and then with horseradish peroxidase-conjugated anti-mouse IgG (KPL, Washington, DC) for one hour. The reactive viral protein was visualized using an ECL WB detection agent (GE Healthcare, Buckinghamshire, UK).

Phylogenetic analysis of capsid, envelope, and nonstructural 1 proteins

All available sequences of DENV-1 to DENV-4, Japanese encephalitis virus, and West Nile virus were downloaded from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/protein) on February 7, 2012. The capsid, envelope, and nonstructural 1 amino acid sequences were extracted from these sequences using the results of a BLAST (Basic Local Alignment Search Tool) homology search[27] against the corresponding proteins. These sequences were aligned using MAFFT version 6.705b[28] after removing redundant sequences. Phylogenetic trees were constructed using the neighbor-joining method[29] with MEGA5.[30] All positions containing gaps and missing data were eliminated.

Results

Preparation of mouse monoclonal antibodies against DENV

To prepare hybridoma clones producing anti-DENV monoclonal antibodies, spleen cells derived from mice immunized with DENV-2 infected cells were fused with myeloma cell lines as previously described.[26] After screening and single-cell cloning, we obtained a total of 60 hybridoma clones specifically reacted with DENV-2-infected Vero cells, but not with uninfected Vero cells, by indirect immunofluorescence assay. The 4G2 monoclonal antibody, available as an anti-flavivirus,[31] was used as a positive control for the immunofluorescence assay. This 4G2 reacted with the envelope protein of DENV in the cytoplasm of infected Vero cells (Figure 1). Of the 60 clones generated in this study, five clones were reacted

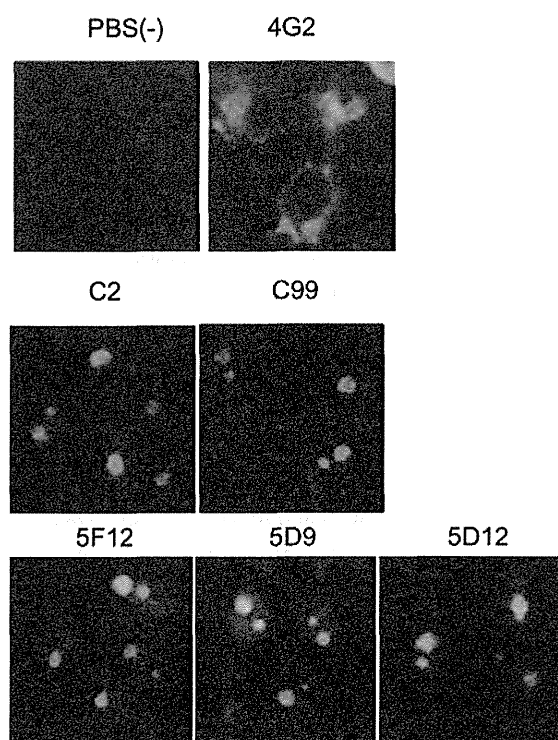

Figure 1 Indirect immunofluorescence assay of anti-DENV-2 reacted with the nuclear protein of DENV-2 infected cells.
Notes: Vero cells were infected with DENV-2 and fixed 48 hours after infection with 4% paraformaldehyde. Cells were stained with the indicated hybridoma culture fluid, C2, C99, 5F12, 5D9 and 5D12, or 4G2 (anti-envelope monoclonal antibody) as a control.

with the nuclei of infected cells (Figure 1). Two clones (C2 and C99) were derived from mice immunized with the brain homogenate of BALB/c suckling mice injected with DENV-2, while three clones (5F12, 5D9, and 5D12) were derived from three mice immunized with B7-cells infected with DENV-2. All the other 55 monoclonal antibodies reacted with the cytoplasm of infected cells (data not shown).

Monoclonal antibodies recognizing DENV capsid protein

To identify a viral protein recognized by five monoclonal antibodies which were shown to stain the nuclei of infected cells as above, a Western blot assay with the culture supernatants of individual hybridoma clones was carried out using the lysate of DENV-2-infected Vero cells that were treated with beta-mercaptoethanol and heated at 100°C. The C2 monoclonal antibody reacted specifically with 12 kDa DENV-2 capsid protein (Figure 2A). To confirm that this binding protein was the capsid protein, Vero cells were transfected with plasmid expressing recombinant DENV-2 capsid protein. This monoclonal antibody reacted with recombinant DENV-2 capsid protein in transfected cells on Western blotting and immunofluorescence assay (Figure 2 and Table 1). Although the other four monoclonal antibodies (C99, 5F12, 5D9, and 5D12) did not react with the 12 kDa capsid protein on Western blot assay (Figure 2A), these monoclonal antibodies similarly reacted with recombinant capsid protein in transfected Vero cells by immunofluorescence assay (Figure 2B). Thus, we concluded that these five monoclonal antibodies recognized the DENV capsid protein.

Cross-reactivity of monoclonal antibodies to DENV capsid protein

To determine the cross-reactivity of the five above-mentioned monoclonal antibodies to DENV capsid protein with each serotype of DENV and Japanese encephalitis virus, individual monoclonal antibodies in culture supernatants were examined by immunofluorescence assay using Vero cells infected with DENV-1 to DENV-4, as well as Japanese encephalitis virus (Figure 3). The cross-reactivities of our five monoclonal antibodies are summarized in Table 1. Three monoclonal antibodies, C2, 5F12, and 5D9, were reacted specifically with only DENV-2 but not with the other serotypes, whereas the other two monoclonal antibodies, C99 and 5D12, were cross-reacted with DNEV-2 and DENV-4. None of these five monoclonal antibodies showed cross-reactivity with DENV-1 or DENV-3. In addition, there were no positive reactions with these five monoclonal antibodies in Vero cells infected with Japanese encephalitis virus (Figure 3 and Table 1).

Phylogenetic analysis of flavivirus

Examination of anti-DENV capsid protein monoclonal antibodies for their cross-reactivity among four serotypes of DENV, as well as Japanese encephalitis virus and West Nile virus belonging to the same flavivirus, revealed only limited cross-reactivity. Therefore, we examined the situations for individual serotypes of DENV as well as Japanese encephalitis virus and West Nile virus by analysis of phylogenetic trees. The sequences of flavivirus capsid, envelope, and nonstructural 1 proteins all derived from the National Center For Biotechnology Information database were used for construction of individual phylogenetic trees. The numbers of the amino acid sequences used for phylogenetic analysis were shown in Table 2.

As observed in the cross-reactivity of monoclonal antibodies, phylogenetic analysis of the capsid protein showed that DENV-2 and DENV-4 were clustered in the same branch, while DENV-1 and DENV-3 were clustered in the other branch (Figure 4A, capsid protein). This meant that the capsid protein of DENV-2 was most closely related

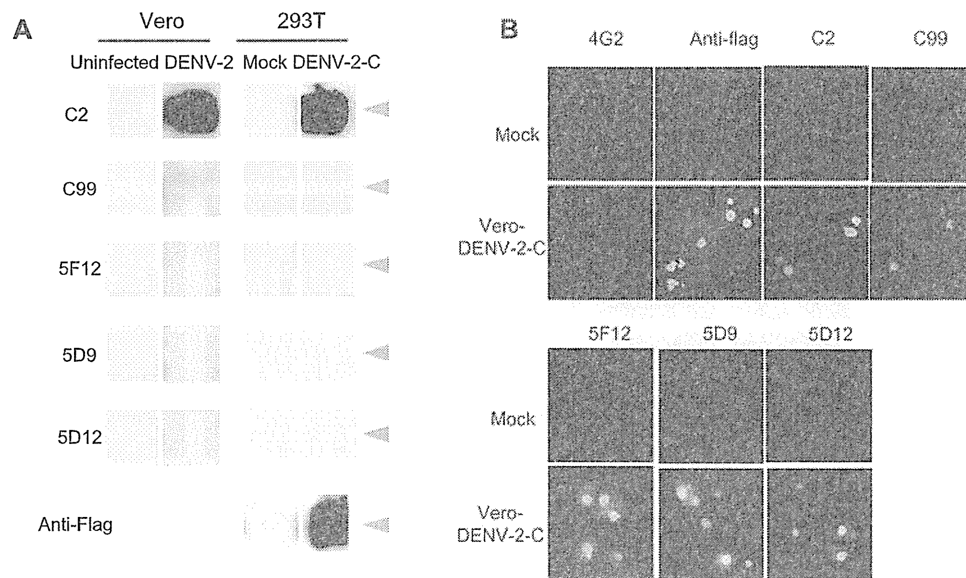

Figure 2 Identification of antigenic DENV-2 capsid protein. (**A**) Lysates of Vero cells infected with DENV-2 and 293T cells transfected with the DENV-2-C expression plasmid encoding Flag were used as antigens. Their reactivity against various antibodies was analyzed by Western blotting. Anti-Flag antibody was used as a positive control of capsid protein expression. Arrowheads showed 12 kDa. (**B**) Indirect immunofluorescence assay of monoclonal antibodies using Vero cells transfected with the DENV-2 capsid protein expression plasmid and fixed 48 hours following infection with 4% paraformaldehyde.
Note: Cells were stained with each monoclonal antibody and anti-Flag antibody, followed by Alexa Fluor® 488 goat anti-mouse IgG secondary antibody.

phylogenetically to that of DENV-4, but very far from those of DENV-1 and DENV-3. On the other hand, phylogenies of the envelope and nonstructural 1 proteins showed different branching orders among the four serotypes, with DENV-4 being the first diverge, followed by DENV-2, and the final diverge being between DENV-1 and DENV-3 (Figure 4, envelope and nonstructural 1 proteins). The distances between DENV clusters seem to be comparable with those between Japanese encephalitis virus and West Nile virus (Figure 4). Furthermore, clusters in the flavivirus capsid protein showed more diversity than those in the flavivirus envelope and nonstructural 1 proteins (Figure 4).

Discussion

In this study, we produced mouse monoclonal antibodies to the capsid protein of DENV. Five hybridoma cells producing anti-capsid monoclonal antibodies were successfully generated and characterized. Interestingly, none of the antibodies generated could bind with all serotypes of DENV. Consequently, this result was greatly different from our initial expectations. Phylogenetic tree analysis using database-derived sequences of DENV-1 to DENV-4 supported the above result, because capsid protein variations were higher than our initial expectation, as confirmed by comparison with other viral proteins, such as the envelope and nonstructural 1 proteins.

By immunization of BALB/c mice with DENV-2 antigens, we obtained only five monoclonal antibodies reactive with DENV capsid protein among the 60 monoclonal antibodies. Similarly, it has been reported that only a few anti-DENV capsid protein monoclonal antibodies are obtained by immunization with the whole virus.[32] In this study, the C2 monoclonal antibody reacted with DENV capsid protein by both Western blotting and immunofluorescence assays,

Table 1 Summary of cross-reactivity of MAb within *Flavivirus* and specificity MAb against DENV-2-C protein

	DENV-1*	DENV-2	DENV-3	DENV-4	JEV	DENV-2-C plasmid**
4G2	+	+	+	+	+	–
C2	–	+	–	–	–	+
C99	–	+	–	+	–	+
5F12	–	+	–	–	–	+
5D9	–	+	–	–	–	+
5D12	–	+	–	+	–	+
Anti-Flag	ND	ND	ND	ND	ND	+

Notes: *DENV-1 to -4 and JEV infected Vero cell; **DENV2-C protein expression plasmid transfected Vero.
Abbreviations: +, positive; –, negative; ND, not done.

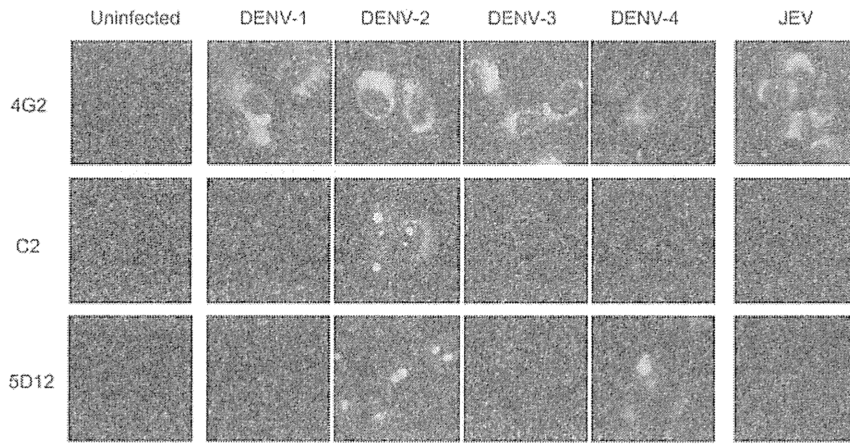

Figure 3 Determination of cross-reactivities of monoclonal antibodies to flavivirus.
Notes: Vero cells, infected with each DENV serotype (1 to 4) and Japanese encephalitis virus, or uninfected Vero cells were stained using the indicated monoclonal antibodies with Alexa Fluor® 488 goat anti-mouse IgG secondary antibody. The cross-reactivities of other monoclonal antibodies are summarized in Table 1.

while the C99, 5F12, 5D9, and 5D12 monoclonal antibodies reacted with the capsid protein only on immunofluorescence assay. These results suggest that the C2 monoclonal antibody recognized a linear epitope, and other monoclonal antibodies recognized conformational epitopes, and the structure of these conformation epitopes might be lost by cell-lysis buffer treatment. Further work is needed on the structural analysis of this epitope.

Three monoclonal antibodies were specific to DENV-2 and the other two clones were cross-reactive with DENV-2 and DENV-4. Previous research on anti-DENV capsid protein has reported similar results for serotype specificity and/or low cross-reactivity with DENV capsid protein.[6,32–34] However, the reason for the low cross-reactivity of DENV capsid protein has not been discussed in these papers. Our bioinformatics characterization of DENV capsid protein sequences from the database revealed that DENV-2 and DENV-4 were clustered in the same branch, while DENV-1 and DENV-3 clustered in the other branch (Figure 4). However, these classifications of DENV capsid protein were not correlated with those of the DENV envelope and nonstructural 1 proteins. In general, epitopes recognized with antibodies are short amino acid sequences or protein conformations; therefore, it may be difficult to discuss serological cross-reactivity using only phylogenetic trees from amino acid sequences. However, our bioinformatics characterization results for the capsid protein may correlate well with the results for anti-capsid monoclonal antibodies obtained by immunization with DENV-2 antigens

Table 2 Number of amino acid sequences of *Flavivirus* extracted from NCBI database

	Capsid	Envelope	NS1
DENV-1	116	805	275
DENV-2	101	689	242
DENV-3	90	391	181
DENV-4	32	217	44
WNV	64	245	124
JEV	60	353	65

Abbreviations: DENV, Dengue virus; WNV, West nile virus; JEV, Japanese encephalitis virus.

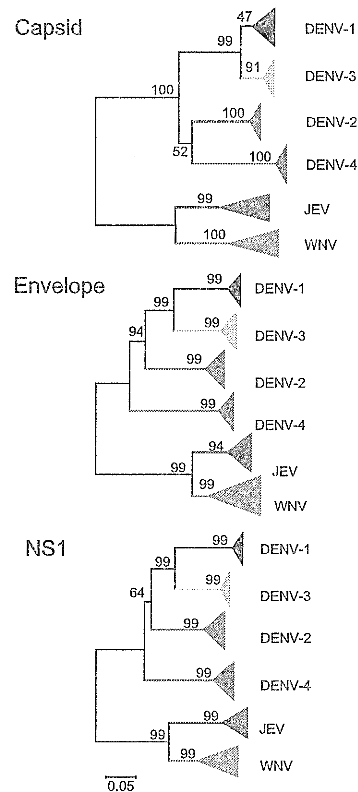

Figure 4 Phylogenetic trees of flavivirus capsid, envelope, and nonstructural 1 protein inferred using the neighbor-joining method.
Notes: All positions containing gaps and missing data were eliminated. Bootstrap values obtained from 500 replicates were shown on major branches. DENV-1 to DENV-4 strains are shown in black, red, light green, and blue, respectively. West Nile and Japanese encephalitis viruses are shown in dark green, and purple, respectively.

cross-reactive with DENV-2 and DENV-4, but not with those for DENV-1 and/or DENV-3. The phylogenetic tree for the flavivirus capsid proteins also showed that the distance between DENV-2 and DENV-4 clusters and between DENV-1 and DENV-3 clusters was comparable with that between Japanese encephalitis virus and West Nile virus. This bioinformatics information suggests that DENV-2 capsid antibodies that are cross-reactive with DENV-4 are relatively frequent, whereas those that are cross-reactive with DENV-1 and/or DENV-3 are rare (Table 1). The diversity between DENV serotypes was comparable with those between Japanese encephalitis virus and West Nile virus (Figure 4). These tendencies were correlated with those not only in capsid proteins, but also in other proteins such as envelope and nonstructural 1 proteins. Our results show that phylogenetic distances between the four serotypes of DENV were as different as that of Japanese encephalitis virus and West Nile virus. However, the diversity of flavivirus capsid protein was much higher than that for envelope and nonstructural 1 proteins. Therefore, these data suggest that there may be difficulty in establishing monoclonal antibodies against DENV capsid protein that are cross-reactive with all the subtypes of DENV. The monoclonal antibodies specific for DENV-2 generated in this study could be used for serotyping of DENV infection. However, DENV capsid protein can be used for serotyping of DENV infection but not for rapid diagnostic test kits to detect all four DENV serotypes.

The levels of diversity among the DENV serotypes reflect the differences between species of flavivirus. This high diversity of DENV correlates with the classification of individual DENV serotypes. However, it is well known that there is significant diversity among genotypes for the individual DENV serotypes.[35,36] These results suggest that individual DENV serotypes have evolved in their own particular way, unlike other flaviviruses.

In this study, the monoclonal antibodies generated specifically for DENV-2 capsid protein can be used for serotyping of DENV infection and for early diagnosis of high titers of viremia in patients with hemorrhagic Dengue fever or Dengue shock syndrome caused by DENV-2 infection. We could not obtain monoclonal antibodies cross-reactive with all four serotypes using mice immunized with a sole serotype of DENV. Our phylogenetic analysis of DENV and the high diversity of the DENV capsid protein sequence support this conclusion.

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Disclosure

The authors report no conflicts of interest in this work.

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SHORT REPORT

Significant neutralizing activities against H2N2 influenza A viruses in human intravenous immunoglobulin lots manufactured from 1993 to 2010

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Abstract: Influenza A H2N2 virus, also known as the Asian flu, spread worldwide from 1957 to 1967, although there have been no cases reported in humans in the past 40 years. A vaccination program was introduced in Japan in the 1960s. Older Japanese donors could have been naturally infected with the H2N2 virus or vaccinated in the early 1960s. Human intravenous immunoglobulin (IVIG) reflects the epidemiological status of the donating population in a given time period. Here, the possible viral neutralizing (VN) activities of IVIG against the H2N2 virus were examined. Hemagglutination inhibition (HI) and VN activities of IVIG lots manufactured from 1993 to 2010 in Japan and the United States were evaluated against H2N2 viruses. High HI and VN activities against H2N2 viruses were found in all the IVIG lots investigated. HI titers were 32–64 against the isolate in 1957 and 64–128 against the isolates in 1965. VN titers were 80–320 against the isolate in 1957 and 1280–5120 against the isolates in 1965. Both the HI and VN titers were higher against the isolate in 1965 than in 1957. Thus, antibody titers of IVIG against influenza viruses are well correlated with the history of infection and the vaccine program in Japan. Therefore, evaluation of antibody titers provides valuable information about IVIGs, which could be used for immune stimulation when a new influenza virus emerges in the human population.

Keywords: IVIG, influenza, H2N2, neutralization

Introduction

The highly pathogenic avian influenza A H5N1 virus has spread among wild birds worldwide. As of April 2012, there have been 602 cases of human infections, with an extremely high mortality rate of about 59%.[1] Therefore, there are public health concerns regarding the possible global emergence of an H5N1 pandemic virus. However, a swine-origin pandemic influenza A H1N1 virus suddenly emerged in 2009. This novel virus spread among human populations within a short period of time because of the low level of immune responses against the virus, especially among young people. Thus, a pandemic influenza virus may be transmitted to humans because of limited immune responses against the virus in humans. The H2N2 virus could be considered one such virus, because it was prevalent in humans between 1957 and 1967, and a vaccination program was introduced in Japan in the 1960s.[2] However, although H2N2 continues to circulate among birds and pigs, this virus has not infected humans for the last several decades.[3] Based on the above background, it might be worthwhile examining the neutralizing activity of human intravenous immunoglobulin (IVIG) against the H2N2 virus.

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Generally, IVIGs are manufactured in individual lots with serum donations from over 10,000 healthy donors. Therefore, IVIG contains various antibodies against numerous human pathogens including seasonal influenza viruses. In fact, not only current (manufactured in 2008) but also previous (manufactured in 1999) IVIGs contain antibodies with significant neutralizing titers against seasonal and pandemic 2009 influenza viruses.[4] The antibodies comprising IVIG therefore reflect the epidemiological status of the donating population, in a particular time period and geographical area.

In this study, IVIG lots manufactured from 1993 to 2010 were evaluated for hemagglutination inhibition (HI) and virus neutralizing (VN) activities against the H2N2 virus.

Material and methods

Clinical isolates of H2N2 (A/Okuda/1957, A/Izumi/5/1965, A/Kaizuka/2/1965), IVIGs manufactured from 1993 to 2010 from healthy donors in Japan (currently Kenketsu Venoglobulin®-IH; Benesis Corporation, Osaka, Japan), and IVIGs manufactured in 1993 and 1999 from healthy donors in the United States were used in this study (Table 1).

The viruses were propagated in Madin–Darby canine kidney (MDCK) cells or in the allantoic cavity of 11-day-old embryonated chicken eggs. The culture media and allantoic fluids were stored at −80°C prior to use. Viral infectivity (FFU/mL) was titrated in MDCK cells using the peroxidase-antiperoxidase (PAP) staining technique.[5] The HI test using 0.7% guinea pig erythrocytes was carried out as described previously.[5] HI titers are expressed as the reciprocal of the highest dilution of the IVIG preparation showing inhibition. The VN test was also carried out as described previously.[5] Briefly, each IVIG was serially diluted two-fold with serum-free minimal essential medium. The IVIG dilutions (30 μL of each) were mixed with 100 FFU (30 μL) of virus. After incubation for 30 minutes at 37°C, the mixture (30 μL) was applied to MDCK cells in a 96-well microplate. After incubation for 16 hours, the cells were fixed with ethanol and stained using PAP technique. The results are expressed as the reciprocal of the dilution resulting in 50% neutralization (VN_{50}).

Table 1 Cross-reactivity of intravenous immunoglobulins to the influenza H2N2 virus

IVIG	H2N2 virus					
	Okuda/1957		Izumi/5/1965		Kaizuka/2/1965	
	HI	VN_{50}	HI	VN_{50}	HI	VN_{50}
1993JP, Lot A	32	160	64	1280	64	1280
1993US, Lot B	64	320	128	5120	128	2560
1999JP-A, Lot C	32	160	64	1280	64	1280
1999JP-B, Lot D	32	80	64	1280	64	1280
1999JP-C, Lot E	32	160	64	1280	64	1280
1999JP-D, Lot F	32	160	64	1280	64	1280
1999US, Lot G	64	320	128	5120	128	2560
2000JP, Lot H	32	160	64	1280	64	1280
2003JP, Lot I	32	80	64	1280	64	1280
2006JP, Lot J	32	160	64	1280	64	1280
2007JP, Lot K	32	160	64	1280	64	1280
2008JP, Lot L	32	160	64	1280	64	1280
2009JP, Lot M	32	80	64	1280	64	1280
2010JP, Lot N	32	80	64	1280	64	1280

Abbreviations: HI, hemagglutination inhibition titer; IVIG, intravenous immunoglobulin; JP, Japan; US, United States; VN_{50}, virus neutralization titer giving 50% inhibition of infectivity.

Results

As summarized in Table 1, the titers were significant against all three isolates of H2N2: HI titers of 32–64 and VN_{50} titers of 80–320 against Okuda/1957, HI titers of 64–128 and VN_{50} titers of 1280–5120 against Izumi/5/1965, and HI titers of 64–128 and VN_{50} titers of 1280–2560 against Kaizuka/2/1965. Both the HI and VN_{50} titers were higher against the isolate in 1965 than in 1957 (Table 1). There were no apparent differences in the HI and VN_{50} titers against H2N2 virus isolates among the IVIG products prepared in the United States and Japan in different years.

Discussion

Currently, most of the donor population has not been exposed to the H2N2 virus. IVIG is manufactured from pooled plasma. In a previous study, HI and VN activities against seasonal influenza H1N1 and H1N1-pdm2009 were examined in IVIG lots manufactured from 1999 to 2008. The IVIGs indicated high and stable HI and VN activity against H1N1. It was taken into account that the donor population may have been immunized through native infections and/or vaccine programs. Interestingly, the IVIGs also showed low but significant HI and VN activities against H1N1-pdm2009 despite most of the donor population never having been exposed to swine H1N1 or the Spanish flu.[4]

In this study, HI and VN activities against the H2N2 virus, which has not been included in vaccine programs for the past 45 years, were measured in IVIGs manufactured from 1993 to 2010 in Japan. Both the HI and VN_{50} titers were higher against H2N2 isolates in 1965 than in 1957. It has been reported that people under the age of 50 years have little or no immunity to H2N2 and that people older than 50 years who have been exposed to the virus show a higher rate of resistance.[6] Therefore, older Japanese donors could have been naturally infected with the H2N2 virus or vaccinated in the

early 1960s. Consequently, antibody titers against the H2N2 virus in the general population will decrease in the future, which could lead to an H2N2 pandemic. It is not clear why such differences of HI and VN activities of IVIG preparations were observed between the United States and Japan. One possible explanation may be derived from the difference in environmental factors and population for blood donation between the two countries.

Conclusion

The results suggest that the antibody titers against influenza viruses in IVIGs correlate with the history of infection and with vaccination programs. Therefore, the evaluation of antibody titers provides valuable information about IVIGs, which could be used for immune stimulation when a new influenza virus emerges in the human population.

Acknowledgments

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Disclosures

Yoshinobu Okuno and Ritsuko Kubota-Koketsu are employed by The Research Foundation for Microbial Diseases of Osaka University. Mikihiro Yunoki is employed by the Benesis Corporation. All other authors report no conflicts of interest in this work.

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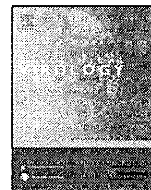

Analysis of Vis–NIR spectra changes to measure the inflammatory response in the nasal mucosal region of influenza A and B virus-infected patients

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ABSTRACT

Background: Human influenza A and B viruses cause severe seasonal respiratory tract infections, especially in infants and young children. Influenza A and B viruses have been reported to produce different symptoms and/or severity in infected patients, although these remain inconclusive.
Objectives and study design: In this study, non-invasive visible and near-infrared (Vis–NIR) spectroscopy was used for comparative analysis of the inflammatory response to influenza A and B virus infections, by measuring changes in water peak (970 nm) spectra collected from patient nasal mucosal regions.
Results: The results suggested that infection with influenza B virus induced more severe inflammatory responses in the nasal mucosal region than influenza A virus.
Conclusions: These are the first data showing different inflammatory responses to influenza A and B viruses at the sites of virus infection.

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1. Background and objectives

Temperature is an important indicator of circadian rhythms, and physical and mental activities. Body temperature can reflect an abnormality in blood flow or fever as a result of an inflammatory response, and provide useful diagnostic information. For example, breast cancer screening can be performed by detection of differential temperatures of 1 °C[1] or 2.5 °C.[2] Malignant tumors show a 1–2 °C increase in temperature compared to the temperature of arterial blood.[3] In contrast, a high temperature of about 43 °C is essential for cancer therapy.[4]

Thermography can be used to analyze surface and body temperature, but cannot quantify body temperature due to its low accuracy. Nuclear magnetic resonance (NMR) spectroscopy, microwave radiometry,[5] and ultrasound thermometry[6] can also detect temperature change. However, none of these methods is applicable to local or regional temperature changes. In contrast, near-infrared radiation (NIR) transmitted through the body can be used for determining temperature changes in a small body region.

Recently, the potential of visible and near-infrared (Vis–NIR) spectroscopy for virus diagnosis has been reviewed.[7] Vis–NIR spectroscopy measures absorption of near-infrared radiation. Oxyhemoglobin, deoxyhemoglobin, cytochrome c oxidase, and water are the major biological molecules absorbed by Vis–NIR radiation.[7] Water is especially sensitive to temperature changes, showing an increase in Vis–NIR absorption and a peak shift. In this study, Vis–NIR spectra was analyzed to investigate the inflammatory response in the nasal mucosal region of patients with non-influenza virus, influenza A virus, and influenza B virus infections.

2. Study design

2.1. Patients and nasal fluid collection

Clinical nasal aspirates were collected from 244 pediatric patients at the Baba pediatric clinic during the 2006–2007 influenza season (December 26, 2006 to March 31, 2007). The collection method was described previously.[8] Briefly, saline was introduced into the nasal cavity, and fluid was collected using a Belvital nasal aspirator (Melisana, Nogent-sur-Marne, France). To remove cell debris, the nasal fluid was filtered using a stainless steel mesh with 200 grids per inch (25.4 mm). The filtered nasal aspirates were analyzed by immunochromatography for influenza A and B viruses as described below.

Abbreviations: NA, neuraminidase; NIR, near-infrared; NMR, nuclear magnetic resonance; PCA, principal component analysis; SNV, standard normal variate; Vis–NIR, visible and near-infrared.

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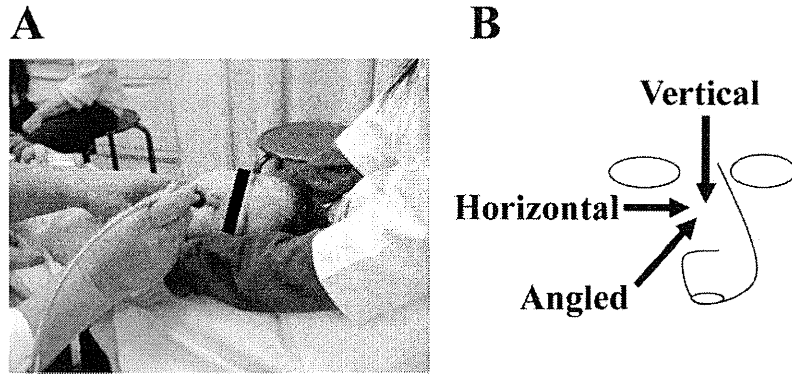

Fig. 1. Directions of Vis–NIR spectra collection from the nasal mucosal region. (A) Picture of representative patient during Vis–NIR spectra collection at the angled direction. (B) Schematic showing the three directions for Vis–NIR spectra collection: angled, vertical, and horizontal.

An axillary electric thermometer C202 (Thermo Corp., Tokyo, Japan) was used for measurement of patient central core temperature.

This research project was approved by the Ethics Committee of the Research Institute for Microbial Diseases of Osaka University and written informed consent was obtained from all children's parents.

2.2. Immunochromatography

Immunochromatography for influenza A and B virus nucleoprotein was performed using a Quick-S Influ A/B kit (Denka Seiken Co. Ltd., Tokyo, Japan) or a Capilia Flu A+B kit (Alfresa Pharma Corporation, Osaka, Japan). The sensitivity and specificity of these commercial antigen detection kits for influenza A in Japan were

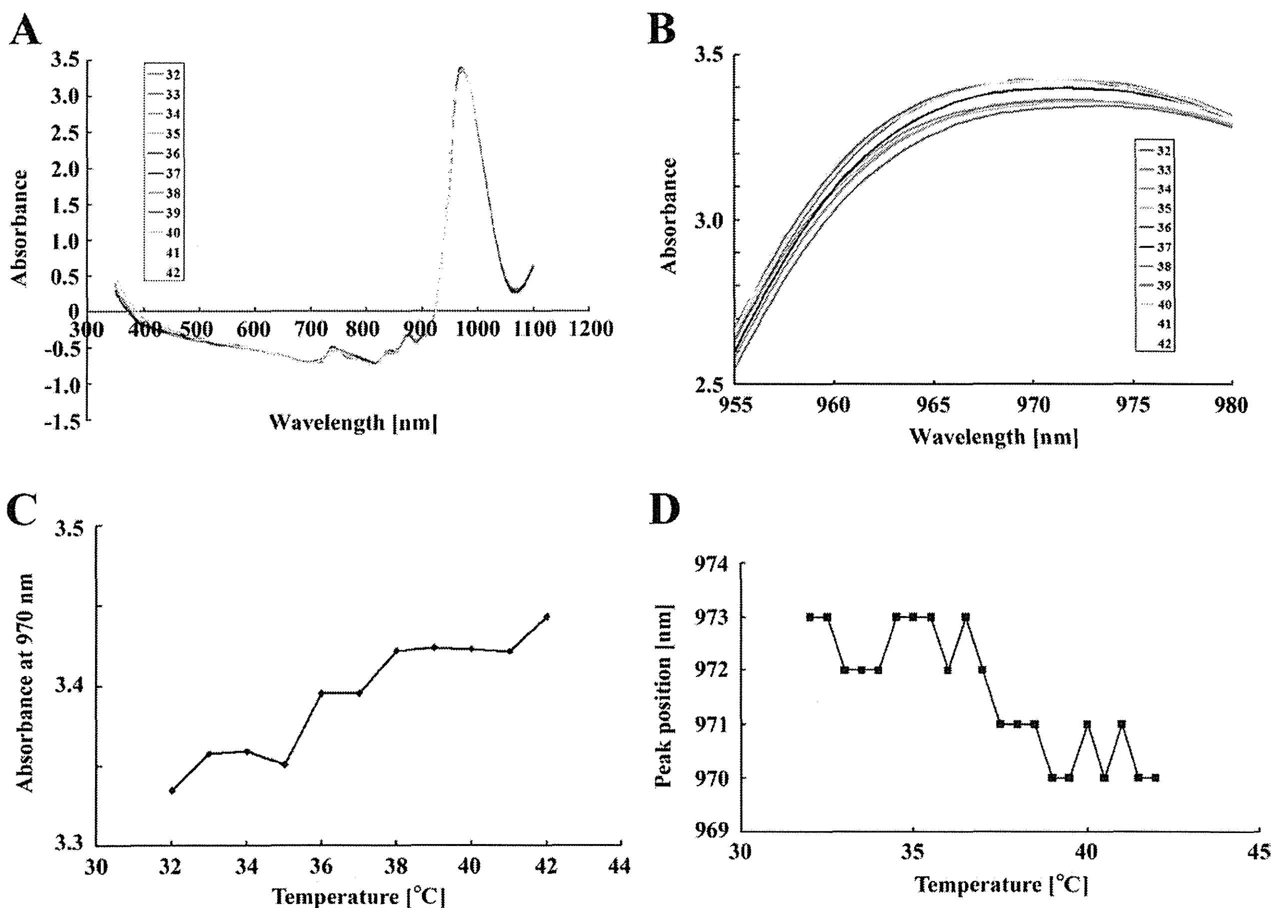

Fig. 2. Temperature dependence of the water Vis–NIR spectra. Water spectra were measured as a function of temperature, with the water temperature controlled by a water bath. The collected spectra were transformed by smoothing and SNV correction in wavelength region (A) 350–1100 nm and (B) 955–980 nm. A sharp peak around 970 nm was observed in the spectra. Temperature-dependent increased absorbance of the peak around 970 nm (C) and blue-shift of this peak (D) was observed.

reported in several studies as follows: 96–98% and 93.9–100%, respectively, for the Capilia Flu A+B kit; and 82.8% and 97.0%, respectively, for the Quick-S Influ A/B kit, respectively.[9] No significant difference in specificity has been reported for influenza A and B viruses. Nasal fluids from the 244 pediatric patients were assayed for influenza A and B viruses by immunochromatography and, based on this analysis, the patients were grouped as non-influenza virus-infected patients, influenza A virus-infected patients, or influenza B virus-infected patients. The age, sex, date, antigen detection test kit results, Vis–NIR measurement time, near-infrared spectra, and axillary temperature were recorded for all patients.

2.3. Vis–NIR measurement and analysis

Vis–NIR spectra of patient noses were measured in three directions (angled, vertical, and horizontal) using a High Sensitivity Multi Channel Photo Detector MCPD-7700 (Otsuka Electronics Co. Ltd., Tokushima, Japan) in the wavelength range 600–1100 nm at a resolution of 2 nm with a coaxial optical fiber probe in the interactance mode (Fig. 1). The nasal mucosal region was chosen for spectra collection because it is the entry site for influenza virus infection. Vis–NIR spectra measurement times were not significantly different among the non-influenza virus-infected, influenza A virus-infected, and influenza B virus-infected patient groups (Table S-1). The spectra were pretreated with smoothing + standard normal variate (SNV) correction by Pirouette software (ver. 3.11; Infometrix, USA). To exclude outlier spectra due to measurement errors, principal component analysis (PCA) was applied to the spectra and those with a >20 Mahalanobis distance were excluded as outliers.

2.4. Temperature dependent change of water spectra

The change of water spectra was determined from 32 °C to 42 °C at intervals of 0.5 °C using an MCPD-7700 in the interactance mode with spectra pretreatment as above.

2.5. Statistical analysis

The absorbance and peak shift of pretreated spectra, in the water-related region (970 nm) and the non-water related region (1070 nm), were compared for the three patient groups using the Kruskal–Wallis test and Dunn's multiple comparison test.

3. Results

We first analyzed the temperature dependence of the water spectrum and found a temperature-dependent increase in absorbance and a peak shift to a shorter wavelength of the peak around 970 nm (Fig. 2).

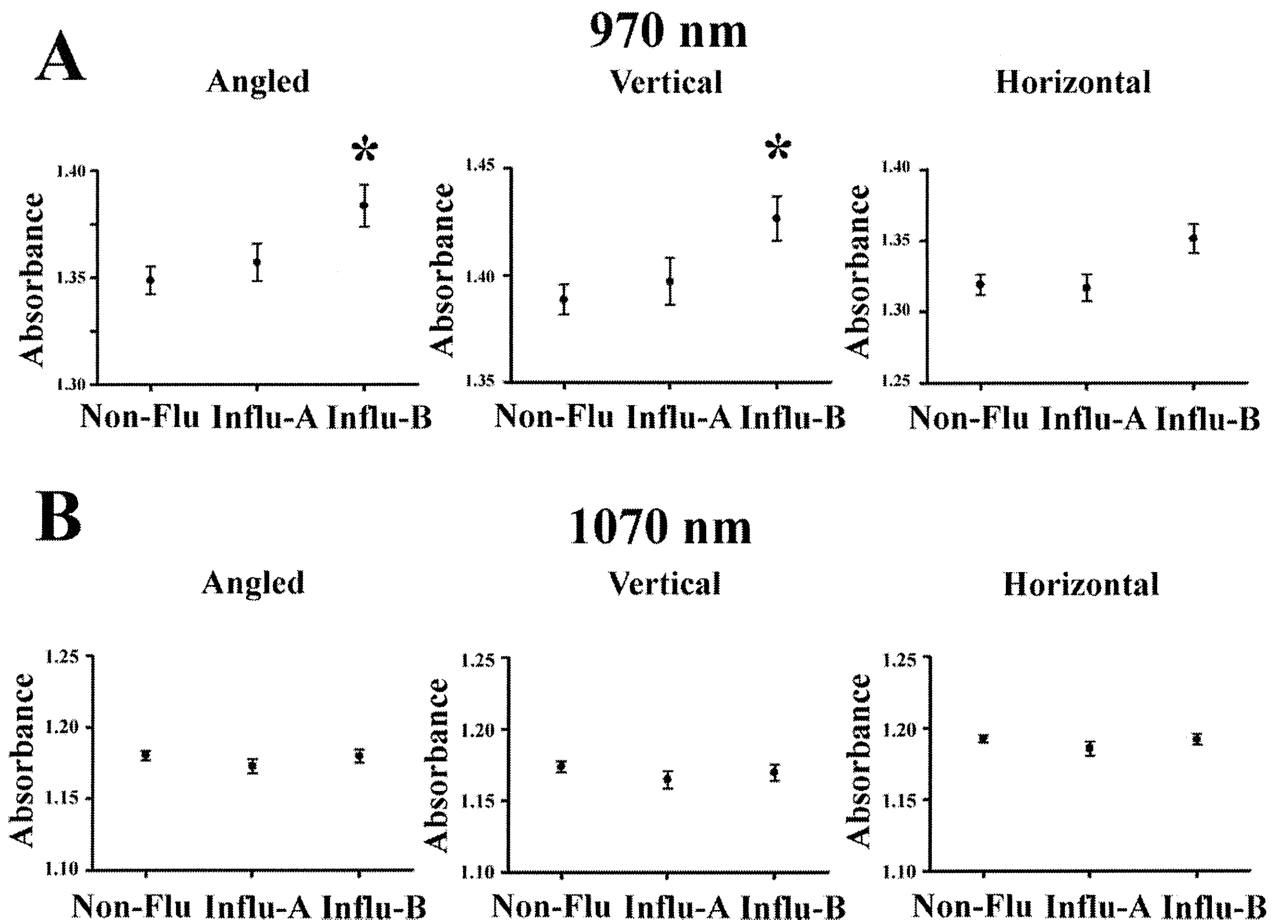

Fig. 3. Absorbance at 970 and 1070 nm in the Vis–NIR spectra collected from patients' noses at angled, vertical, and horizontal directions. Absorbance in the Vis–NIR spectra of nasal mucosal regions of non-influenza virus-infected, influenza A virus-infected, and influenza B virus-infected patients at (A) 970 nm and (B) 1070 nm are shown as the mean ± standard deviation for each patient group. The absorbance at 970 nm of influenza B virus-infected patients, but not of influenza A virus-infected patients, was significantly increased compared to non-influenza virus patients for spectra collected at angled and vertical directions (*$P < 0.01$). There was no significant difference among the three patient groups for absorbance at 1070 nm ($P > 0.01$).

Table 1
Absorbance differences of smoothed and SNV-transformed Vis–NIR spectra at 970 and 1070 nm between influenza B virus-infected and non-influenza virus-infected patients.

Spectra collection direction	Wavelength	
	970 nm	1070 nm
Angled	*	N.S.
Vertical	*	N.S.
Horizontal	N.S.	N.S.

* Significant difference ($P<0.01$) between influenza B virus- and non-influenza virus-infected patient groups; N.S., no significant difference ($P>0.01$) between these two patient groups.

Table 2
Peak position of smoothed and SNV-transformed Vis–NIR spectra of the water peak around 970 nm between non-influenza virus-infected and influenza A and B virus-infected patients.

Spectra collection direction	Peak position (nm) for spectra from		
	Non-influenza virus-infected patients	Influenza A virus-infected patients	Influenza B virus-infected patients
Angled	984.7 ± 0.3	984.3 ± 0.5	983.2 ± 0.4**
Vertical	984.3 ± 0.3	983.0 ± 0.4	983.0 ± 0.4*
Horizontal	985.7 ± 0.3	985.3 ± 0.6	985.0 ± 0.5

* Significant difference ($P<0.01$) between influenza B virus- and non-influenza virus-infected patient groups.
** Significant difference ($P<0.001$) between influenza B virus- and non-influenza virus-infected patient groups.

We then studied the 244 pediatric clinic patients from whom nasal fluids had been collected. Immunochromatography of the nasal fluids showed that these patients formed three groups: 126 had a non-influenza virus infection, 50 had an influenza A virus infection, and 68 had an influenza B virus infection. Patient age and core body temperature were not significantly different among the three patient groups (Tables S-2 and S-3).

Absorbance at 970 nm in the Vis–NIR spectra collected from patients' noses at two directions (angled and vertical) was significantly different between influenza B virus-infected patients and non-influenza virus-infected patients, but was not significantly different between influenza A virus-infected patients and non-influenza virus-infected patients (Fig. 3A and Table 1). The peak shift around 970 nm was generally consistent with these results (Table 2): the only significant peak shift was for spectra collected at angled and vertical directions, not at the horizontal direction, suggesting that the collection direction relative to the nasal mucosa was important for detecting water spectra changes. However, absorbance at 1070 nm, which is at the bottom of the peak around 970 nm, was not significantly different among the three patient groups (Fig. 3B and Table 1). Therefore, these studies indicated that influenza B virus infection induced an absorption increase and a peak shift around 970 nm, which may be related to fever (i.e., an inflammatory response) in the nasal mucosal region.

4. Discussion

Temperature is one of the most important indicators of a patient's clinical status. Influenza viruses cause fever, which could be a rapid marker for screening influenza diseases. Virus infection usually induces an inflammatory response against the virus, resulting in an increase of body temperature. Viruses have specific host entry sites; e.g., respiratory, digestive, urinary, and genital organs. Therefore, body temperature measured as axillary, tympanic, or rectal temperature does not always indicate a virus-induced inflammatory response. The tissue in which a virus replicates should be a preferable site for measuring temperature. For influenza viruses, the nasal mucosal region should be the best site for measuring temperature, because these viruses infect the respiratory tract.

Fever is thought to be the most common symptom of influenza A and B virus infection. It has been reported that patient temperatures >39 °C were more common with influenza A than with influenza B virus infection,[10] but another study reported that there was no temperature difference between the two groups.[11] Other studies reported that influenza B virus caused less severe fever compared to influenza A virus, but the temperature difference between influenza A and B virus infections in children was relatively small.[11] These results may be due to most influenza in adults being due to a re-infection, while most influenza in children is a first infection. The frequency of digestive tract symptoms is higher in influenza B virus infections than in influenza A virus infections.[12] Other studies have reported that most clinical symptoms are not different between influenza A and B virus infections, although the clinical progression of influenza B virus infection may be slightly slower than influenza A virus infection.[13]

Myalgia, fever, headache, general malaise, and sore throat have been reported to be frequent symptoms in influenza A and B infections,[12] whereas gastrointestinal symptoms (e.g., nasal discharge, abdominal pain, nausea, epigastralgia, and diarrhoea) were more common in influenza B virus infections.[12,14,15] However, such severe symptoms were not observed in another study.[11] Although influenza B virus infections were reported to be less severe and of shorter duration then influenza A virus infections,[16] myalgia was equally prevalent in adult influenza A and B virus-infected patients, in contrast to the influenza B virus clinical picture in children.[17–19] Therefore, it remains inconclusive whether there are differences in symptoms between influenza A and B virus infections.

Since the present study focused on analysis of Vis–NIR spectra collected from influenza A and B patients, we did not analyze patient clinical symptoms in detail. Our only analysis of clinical symptoms showed that fever in the core body analyzed by an axillary electric thermometer was not significantly different between influenza A and B patients, whereas absorbance at a wavelength related to fever, collected at the nasal mucosal region, was significantly different between influenza A and B patients. The comparison further suggested influenza B virus infections induced a more severe inflammatory response in the nasal mucosal region than influenza A virus infections. These are the first data to show different inflammatory responses in a peripheral region between influenza A and B virus infections. Such data could provide a useful approach for development of diagnostic methods for discriminating influenza A and B virus infections. However, as other symptoms also might indicate differential inflammatory responses between influenza A and B infections, further studies on clinical symptoms are necessary. In addition, as the influenza virus titer and the time point after clinical onset might be related to the level of inflammatory response, analysis of the relationship among Vis–NIR spectra, virus titer, and time course after clinical onset are required.

Differentiation of influenza A and B virus infections is essential for choosing anti-influenza drugs for individual patients. There are two types of anti-influenza virus drugs, one inhibiting the influenza virus M2A protein and the other inhibiting influenza virus neuraminidase (NA).[20] Amantadine is an M2A inhibitor, while zanavir and oseltamivir are NA inhibitors. Since influenza B viruses do not have M2A, amantadine is not effective for treating influenza B virus infections. In contrast, since influenza A and B viruses have similar NAs, zanavir and oseltamivir affect influenza virus A and B infections. Therefore, differentiation of influenza A and B virus infections is important for the choice of anti-influenza drugs.

Currently, there are various diagnostic methods for influenza infections, such as immunochromatography, reverse transcription (RT)-polymerase chain reaction (PCR), and real-time RT-PCR. At present, the price of a single immunochromatography test is 10 USD. The time required for an immunochromatography test is 30 min. Since immunochromatography can detect only high virus concentrations, additional methods such as RT-PCR need to be combined for a highly sensitive method for detection of influenza virus. At present the cost of a single real-time or normal RT-PCR is 5–10 USD (10–20 USD for duplicate tests). The time required for a PCR test is 6 h and the price of a PCR instrument is more than 8000 USD. In contrast to these conventional methods, Vis–NIR spectroscopy requires a time (4–5 h) similar to that of PCR to develop a calibration model. Furthermore, a Vis–NIR spectroscopy instrument is expensive (more than 10,000 USD), similar to that of PCR. However, after development of a calibration model, Vis–NIR spectroscopy can be rapid (less than 1 min for a measurement) and requires no regents for the analysis.[7] The price of a single measurement is only the electricity expense. Therefore, if a successful calibration model can be developed, the cost and efficiency of Vis–NIR spectroscopy as an influenza diagnostic method would be excellent. Thus, Vis–NIR spectroscopy combined with a conventional diagnostic method may provide rapid, sensitive, cost-effective influenza diagnosis.

In conclusion, analysis of Vis–NIR spectra around the water absorption peak showed that influenza B virus infections caused severe fever in the nasal mucosal region compared to influenza A virus infections. This method can be used in combination with conventional diagnostic methods for differentiating influenza A and B virus infections. In addition, analysis of Vis–NIR spectra may also be applied to other respiratory infectious diseases. The applicability of this method to different types of viruses (i.e., respiratory syncytial virus and adenovirus) is now being studied.

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Competing interests

None declared.

Ethical approval

This research project was approved by the Ethics Committee of the Research Institute for Microbial Diseases of Osaka University and written informed consent was obtained from all children's parents.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcv.2012.08.015.

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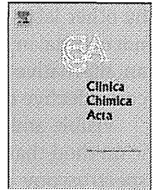

Discrimination of influenza virus-infected nasal fluids by Vis-NIR spectroscopy

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ABSTRACT

Background: Influenza patients show a severe condition of the respiratory tract with high temperature. Efficient treatment of influenza requires early use of oseltamivir, and thus rapid diagnosis is needed. Recently, rapid diagnostic methods such as immunochromatography have been developed; however, immunochromatography is not an optimal technique because it is relatively expensive and has low sensitivity.
Methods: Visible and near-infrared (Vis-NIR) spectroscopy in the region 600–1100 nm, combined with chemometrics analysis such as principal component analysis (PCA) or soft modeling of class analogy (SIMCA), was used to develop a potential diagnostic method for influenza based on nasal aspirates from infected patients.
Results: The Vis-NIR spectra of nasal aspirates from 33 non-influenza patients and 34 influenza patients were subjected to PCA and SIMCA to develop multivariate models to discriminate between influenza and non-influenza patients. These models were further assessed by the prediction of 126 masked measurements [30 from non-influenza patients, 30 from influenza patients and 66 from patients infected with respiratory syncytial virus (RSV)]. The PCA model showed some discrimination of the masked samples. The SIMCA model correctly predicted 29 of 30 (96.7%) non-influenza patients, and 30 of 30 (100%) influenza patients from the Vis-NIR spectra of masked nasal aspirate samples. Nasal aspirates of RSV-infected patients were predicted as 50% non-influenza and 50% influenza by the SIMCA model, suggesting that discrimination between patients infected with influenza virus and those infected with RSV was difficult.
Conclusions: Although the study sample was small and there was difficulty in discriminating between influenza virus and RSV infection, these results suggest that Vis-NIR spectroscopy of nasal aspirates, combined with chemometrics analysis, might be a potential tool for diagnosis of influenza.
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1. Introduction

Influenza viruses are divided into three types (A, B, and C) based on the antigenicity of nucleoprotein (NP) and matrix protein (M1) [1]. The influenza A virus genome encodes two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), for which antigenicity defines 16 distinct HA and 9 distinct NA subtypes [2,3]. Among the various types and subtypes of influenza virus, A/H1N1, A/H3N2 and B have been mainly associated with human infection [1]. Influenza virus infection causes a severe upper respiratory tract infection in infants, young children, and older individuals, with an estimated 3–5 million cases of severe illness and 250,000–500,000 deaths per year worldwide [1].

Currently, influenza virus infection cannot be reliably diagnosed on clinical features alone [4], because it is difficult to differentiate it from other respiratory diseases, such as infection with adenovirus, parainfluenza virus, respiratory syncytial virus (RSV) [5], hemolytic streptococcus [6], and metapneumovirus [7]. In particular, when RSV and influenza epidemics coincide and hospitalization rates are at their highest, the discrimination between RSV infection and influenza virus infection becomes important and difficult [8]. Furthermore, it is important to discriminate viral infection from bacterial infection in order to decrease the misuse of antibiotics, to minimize the emergence of antibiotic-resistant strains of bacteria, and to reduce the length of hospital stay [9].

Several methods for detecting influenza virus have been developed, such as reverse transcription polymerase chain reaction (RT–PCR), enzyme-linked immunoassay (EIA), immunofluorescence assay, rapid antigen test (immunochromatography) and serological tests [10]. In particular, immunochromatography is useful as a rapid diagnostic assay of influenza virus infection and is broadly used for practical diagnosis in the clinic. In addition, although immunochromatography provides a relatively accurate diagnosis with a specificity for influenza of 98.2%, it has low sensitivity (62.3%) [11]. It's positive and negative likelihood ratios are 34.5 and 0.38, respectively, suggesting that a positive test result is unlikely to be a false positive, but a negative result has a reasonable likelihood of being a false negative and should be confirmed

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by other laboratory diagnostic tests. More importantly, this method is not optimal in terms of cost-effectiveness. To overcome these problems, an alternative diagnostic method using an instrument that facilitates cost-effective diagnosis is needed.

Visible and near-infrared (Vis-NIR) spectroscopy is a spectroscopic method that uses visible light and NIR radiation. Moreover, Vis-NIR spectroscopy requires no sample preparation and no reagents [12], resulting in lower costs and less waste. As a result, Vis-NIR spectroscopy is widely used as an analytical technique in the agricultural, pharmaceutical, chemical and petrochemical industries [12,13]. Vis-NIR spectroscopy has also been used in a broad range of clinical applications [12,14].

2. Materials and methods

2.1. Nasal aspirate samples and body temperature measurement

This research project was approved by the Ethics Committee of Osaka University, and written informed consent was obtained from the parents of all influenza, non-influenza and RSV-infected patients before samples were collected.

Clinical nasal aspirates were collected from pediatric patients attending the Baba pediatric clinic (Tables 1, 2). Before the collection, core body temperature was measured by a C202 axillary electric thermometer (Termo Corp., Tokyo, Japan). The nasal aspirate was collected as follows. Saline was introduced into the nasal cavity, then fluid was collected using a Belvital nasal aspirator (Melisana, Nogent-sur-Marne, France). To remove cell debris, the nasal fluid was filtered using a stainless steel mesh [200 grids per inch (25.4 mm)]. The nasal aspirates were subjected to immunochromatography for influenza viruses using a kit (Esprine kit; Fujirebio Inc., Tokyo, Japan). The nasal aspirates from 34 patients with influenza, diagnosed on the basis of immunochromatography, and those from 35 patients not infected with influenza were used as test samples to develop a calibration model for PCA [15] and SIMCA [16] (Table 1). A further 126 measurements comprising 10 samples from non-influenza patients each with n = 3 spectra; 10 samples from influenza patients each with n = 3 spectra; and 22 samples from RSV-infected patients each with n = 3 spectra were masked and used for prediction (Table 2). RSV infection was analyzed by an immunochromatography kit (Bionx Now kit; Eiken Chemical Co. Ltd., Tokyo, Japan).

2.2. Instrument and data collection

Spectral data from nasal aspirates diluted 10-fold with phosphate buffered saline (PBS) was collected as absorbance values [log(1/T)], where T = transmittance in the wavelength range from 600 to 1100 nm. For each sample, three consecutive Vis-NIR spectra were acquired at 2-nm resolution with an FQA-NIRGUN spectrophotometer (Japan Fantec Research Institute, Shizuoka, Japan) at 37 °C (Fig. 1).

2.3. Data processing

Pirouette software (ver. 3.11; Infometrics, Woodinville, WA) was employed for data processing. To minimize differences in spectra caused by baseline shifts and noise, prior to calibration spectral data were mean-centered and transformed by standard normal variates (SNVs) [17] and smoothing based on the Savitsky–Golay algorithm [18]. To identify the predominant absorbance peaks in the spectra, the PCA [15] and SIMCA [16] algorithms were further applied to develop, respectively, PCA and SIMCA models of influenza diagnosis. To visualize the SIMCA approach, we used Coomans plot [19], which plots "class" distances against one other. Coomans plot can assess the classification performance of the SIMCA model by predicting class membership in terms of distance from the model. The critical distance from the model corresponded to the 0.05 level and was defined as the 95% tolerance interval. The mathematical formulas used are available in the Pirouette manual. The difference in core body temperature was evaluated by Kruskal–Wallis test followed by Dunn's analysis. A value of P<0.05 was considered to be statistically significant.

3. Results and discussion

Rapid diagnosis of influenza is important because the efficacy of oseltamivir is maximized by early commencement of treatment. Clinical benefits are seen only when oseltamivir is given within 48 h of the onset of symptoms [20].

Recently, we have demonstrated the possibility of diagnosing various diseases by Vis-NIR spectroscopy using various types of samples such as blood [21–25], cell culture medium [26], and tissues [27–29]. In this study, the potential spectroscopic diagnosis of influenza using nasal aspirates was investigated. Nasal aspirates from influenza and non-influenza patients, as well as those from RSV-infected patients, were subjected to Vis-NIR spectroscopy coupled with multivariate analysis such as PCA and SIMCA, in order to explore a novel method for the diagnosis of influenza.

Discrimination between the nasal aspirates from influenza patients and those from non-influenza patients using test samples was seen in PCA scores using first principal component (PC1) and second principal component (PC2) analysis (Fig. 1A). In addition, the SIMCA model enabled distinct separation of the Vis-NIR spectra of 100 of 105 (95.24%) non-influenza samples and 95 of 102 (93.13%) influenza samples (Table 3). SIMCA analysis using Coomans plot demonstrated that the nasal aspirate classes of "influenza patients" and "non-influenza patients"

Table 1
Test samples used for developing a calibration model[a].

Sample name	Male/ female	Core body temperature [°C]	Sample name	Male/ female	Core body temperature [°C]
N 6	F	36.3	I 7	M	36.4
N 7	F	36.3	I 8	M	36.0
N 8	F	36.3	I 9	M	36.0
N 9	M	36.4	I 10	F	36.3
N 10	F	36.3	I 11	F	36.0
N 11	M	36.6	I 12	F	36.7
N 12	F	36.0	I 13	M	36.3
N 14	F	36.3	I 15	M	36.4
N 15	F	36.3	I 16	M	36.1
N 16	M	36.1	I 19	F	36.1
N 17	F	36.4	I 20	F	36.4
N 18	M	36.1	I 21	M	36.4
N 19	F	36.3	I 22	F	36.4
N 20	M	36.2	I 23	M	36.0
N 21	F	36.5	I 24	F	36.3
N 22	M	36.4	I 25	M	36.1
N 23	F	36.4	I 26	F	36.0
N 24	M	36.4	I 27	F	36.0
N 25	F	36.3	I 28	F	36.3
N 26	M	36.3	I 29	F	36.3
N 27	F	36.3	I 31	M	36.4
N 28	F	36.1	I 32	M	36.1
N 29	M	36.1	I 33	M	36.0
N 31	F	36.6	I 34	M	36.0
N 32	M	36.6	I 35	F	36.0
N 33	F	36.4	I 37	M	36.2
N 34	F	36.4	I 39	M	36.3
N 35	M	36.4	I 40	M	36.4
N 36	M	36.3	I 41	M	36.4
N 37	M	36.2	I 42	M	36.4
N 38	M	36.4	I 43	F	36.3
N 40	F	36.4	I 44	F	36.4
N 41	M	36.1	I 45	F	36.1
N 42	M	36.3	I 47	M	36.6
N 43	F	36.2			

[a] N6-N43 are non-influenza samples, while I7-I47 are influenza samples. Core body temperatures of non-influenza, influenza, and RSV-infected patients at the time when nasal aspirates were collected from patients are shown.