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Three unique Sendai virus antigenic peptides screened from nucleocapsid protein by overlapping peptide array



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ABSTRACT

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Sendai virus (SeV) is strictly monitored in laboratory rodents. Currently, complete virions have been used as antigens in SeV serological tests. However, the complexity of SeV virion antigen limits the accuracy of the diagnostic method. In the current study, complete SeV virion antigen was separated on SDS-PAGE and analyzed, with nucleocapsid protein (NP) showing predominant antigenicity. A peptide array containing overlapping 14-mer peptides covering the entire NP was developed. The array used SeV positive serum and resulted in four antigenic linear peptides being identified, which were located in the carboxyl-terminus of NP. The four peptides were coated on ELISA plates and tested with SeV positive and SeV negative sera, and the antigenicity of three peptides, NP413–428, NP473–490 and NP507–524, was confirmed. Mixture of the three peptides showed comparable sensitivity and better specificity in clinical rat sera ELISA tests compared with complete SeV virion antigen. In conclusion, the three peptides, NP413–428, NP473–490 and NP507–524, would be good candidates as linear antigens for SeV detection.

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1. Introduction

Sendai virus (SeV), a parainfluenza virus of the paramyxovirus family, commonly infects laboratory rodents (Brownstein and Winkler, 1986). SeV infection is usually subclinical, however, SeV infection is immunosuppressive and can have an immediate as well as a long term effect and can cause high morbidity and mortality when combined with exposure to bacterial pathogens (Jakab, 1981; Kay, 1978). For these reasons, SeV is monitored by the routine surveillance of laboratory rodents.

Serological methods, such as ELISA, are used commonly to detect antibodies against SeV. SeV complete virions are used as antigens for these methods (Ertl et al., 1979; Parker et al., 1979; Suzuki et al., 1987). However, the complexity of SeV virion preparations can lead to cross-reactivity of sera antibodies with nonspecific antigens (Ito et al., 1987).

Nucleocapsid protein (NP) is a key structural unit in SeV, which shows predominant antigenicity in reactions with polyclonal anti-SeV mouse sera (Wan et al., 1995). In the current study, peptide array techniques were employed to screen for linear epitopes of NP in SeV, and three peptides were found as candidate SeV-specific linear antigens for SeV detection.

2. Materials and methods

2.1. SeV complete virion antigen and rat sera

SeV complete virions were harvested from BHK-21 cell culture infected with SeV (VR-105, ATCC, Manassas, USA) (Manaker et al., 1961; Roux and Waldvogel, 1982). Briefly, SeV was prepared in the allantoic fluid of chicken embryos before the virus was infected into BHK-21 cells and left for three days to amplify. Once amplified, batches of SeV virions were harvested from the cultures by differential centrifugation. A mixture of rat sera from barrier facilities, which had tested SeV negative using commercial SeV detection ELISA kits (PL-001, Charles River, Wilmington, USA), was used as a SeV negative control. Twenty specific pathogen-free Sprague-Dawley rats were contained at an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited ABSL-2 facility and were intranasally infected with SeV (Garlinghouse et al., 1987). Three weeks after infection, 20 rats were euthanised with a sodium pentobarbital overdose after blood collection. The sera of rats were confirmed as being SeV positive by ELISA. A mixture of sera was used as positive controls.

Animal protocols were approved by the Institutional Animal Care and Use Committee of the Chinese Institute of Laboratory Animal Science. Inoculation and blood collection were carried out under anesthesia with sodium pentobarbital to minimize rat suffering.

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2.2. Western blot analysis of predominate SeV antigens

Protein from SeV virions was separated on 12% SDS-PAGE (12 channel AE-6450 Dual Mini Slab Chamber, ATTO, Tokyo, Japan). Pre-stained molecular weight standards (sm0671, Fermentas, Vilnius, Lithuania) were used to monitor electrophoresis. Electrophoresis was stopped when the red molecular weight standard at 72 kDa reached the middle of the gel. Proteins were then transferred to a nitrocellulose membrane (Millipore, Bedford, USA). Eleven sera samples (200 μ L, 1:100 diluted with PBS and 3% milk) were loaded on the same membrane separately with the help of multi-channel post blot equipment (AE-6195, ATTO, Tokyo, Japan). After 12 h incubation at 4 °C, the membrane was washed three times with PBST (PBS with 0.05% Tween-20) for 10 min. The membrane was then incubated with HRP conjugated polyclonal goat anti-rat IgG antibodies (1:20000 dilution; Jackson ImmunoResearch, West Grove, USA) for 1 h at room temperature. After washing three times with PBST, the nitrocellulose membrane was visualized using ECL (Millipore, Bedford, USA) and exposed to X-ray film (Kodak, Rochester, USA).

2.3. Peptides array

A set of overlapping 14-mer peptides, obtained by shifting two amino acids from the amino-terminus to the carboxyl-terminus of SeV NP (NP_056871.1), were synthesized on derivatized cellulose membranes using an auto spot peptide synthesizer (Frank, 2002; Frank and Overwin, 1996; Martens et al., 1995). Two hundred and sixty-four peptides were synthesized on the membranes which were consistent with the peptides coding the amino acids of SeV NP.

Peptide array membranes were blocked with blocking buffer (4% skim milk and 5% sucrose in PBST) for 4 h at room temperature. SeV positive and SeV negative rat control sera were diluted to 1:500 with blocking buffer and incubated overnight at 4 °C with two peptide array membranes. The membranes were then washed three times with PBST for 10 min before being incubated with HRP conjugated polyclonal goat anti-rat IgG antibodies (1:20000 dilution) for 1 h at room temperature. After washing three times with PBST, specific bindings on the membranes were detected by ECL. Visualization of chemiluminescence of each spot was performed using an imaging system (Bio-rad, Richmond, USA).

2.4. ELISA analysis of the antigenicity of candidate peptides

SeV ELISA kits (PL-001; Charles River, Wilmington, USA) were used according to the manufacturer's instructions. For comparisons of sensitivity between SeV linear peptides and complete virions, candidate antigenic peptides were synthesized and separately coated on 96-well EIA/RIA plates (Costar, Cambridge, USA). This process was performed as follows. SeV virion antigens or linear peptides were diluted to 10 μ g/mL using coating buffer (28.6 mM of Na_2CO_3 and 71.4 mM of NaHCO_3 , pH 9.6) and distributed (100 μ L/well) on ELISA plates. A mixture of peptides (10 μ g/mL of each peptide) was coated on another plate. Plates were kept at 4 °C overnight. After washing three times with PBST, wells were blocked with blocking solution (PBST with 1% BSA) at room temperature for 30 min before sera samples (1:40 diluted with blocking solution, 100 μ L/well) were added and left to incubate for 1 h at 37 °C. Sera samples were then aspirated off and wells washed three times with PBST. HRP conjugated polyclonal goat anti-rat IgG antibodies dissolved in blocking solution were added (100 μ L/well) and incubated for 1 h at 37 °C. After washing in the same manner, TMB (3,3',5,5'-tetramethylbenzidine) was distributed (100 μ L/well) and plates were incubated at room temperature for 10 min. The enzyme reaction was terminated by the

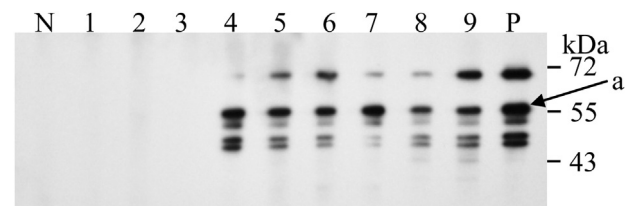


Fig. 1. Western blotting results of SeV complete virions reacted with SeV negative control serum (N), positive control serum (P) and clinical rat sera (1–9). Lanes 1–3 are SeV negative sera and lanes 4–9 are SeV positive sera. Pre-stained molecular weight standards (sm0671, Fermentas, Vilnius, Lithuania) were used. Arrow points to the band at 57 kDa.

addition of 2 M of H_2SO_4 (50 μ L/well), and blanked absorbance values at 450 nm were measured. The ELISA cut-off value was set as the absorbance of the negative control serum at 450 nm (N) plus 0.15 ($N + 0.15$) on SeV complete virion-coated plates.

Those samples with an absorbance value over $N + 0.15$ were regarded as positive and those below $N + 0.15$ were regarded as negative (a judgment criteria at the institute where the study was conducted). The cut-off value in the SeV complete virion-coated plates was also applied in the peptide mixture-coated plates. Each sample was analyzed in triplicate. A Student's *t*-test was used to compare the absorbance of SeV negative samples from the two kinds of ELISA. A *p* value of <0.05 was regarded as statistically significant.

3. Results

3.1. NP is the predominant antigen of SeV with rat anti-SeV sera

The results from Western blot analysis of SeV complete virions showed that no specific band was able to be detected when membranes were incubated with SeV negative control rat serum (N in Fig. 1). Some SeV-specific bands were detected when membranes were incubated with SeV positive control rat serum (P in Fig. 1). Some clinical sera samples (1–9 in Fig. 1) were also tested by western blotting. Reactions with SeV negative sera (1–3 in Fig. 1) did not show any specific band. Reactions with SeV positive sera (4–9 in Fig. 1) showed the 57 kDa band as being the most dominant antigenic band (arrow a in Fig. 1). The molecular weight of the 57 kDa protein was consistent with that of NP of SeV (Buchholz et al., 1993; Ogino et al., 1999; Wan et al., 1995).

3.2. Candidate linear antigenic peptides are located in the carboxyl-terminus of NP

Complete NP overlapping 14-mer peptides arrays were performed on two membranes in the same manner. Membrane reactions with SeV negative and SeV positive control sera are shown in Fig. 2. Peptides at membrane sites 212, 229, 230, 231, 232, 241, 242, 243, 258 and 259 showed higher avidity with SeV positive serum (Fig. 2B) than with SeV negative serum (Fig. 2A). The following sites represent the amino acids of NP in SeV. Site 212 represents amino acids 415–428, sites 229–232 represent amino acids 449–468, sites 241 and 242 represent amino acids 475–490, and sites 258 and 259 represent amino acids 507–522. These linear peptide sequences were located in the carboxyl-terminus of NP.

3.3. Three linear peptides showed antigenicity during ELISA

Four peptides were synthesized according to NP amino acids 413–428, 449–468, 473–490, and 507–524 (Table 1). These four peptides and the complete virions of SeV were coated separately on ELISA plates. Reactions of SeV negative and SeV positive sera

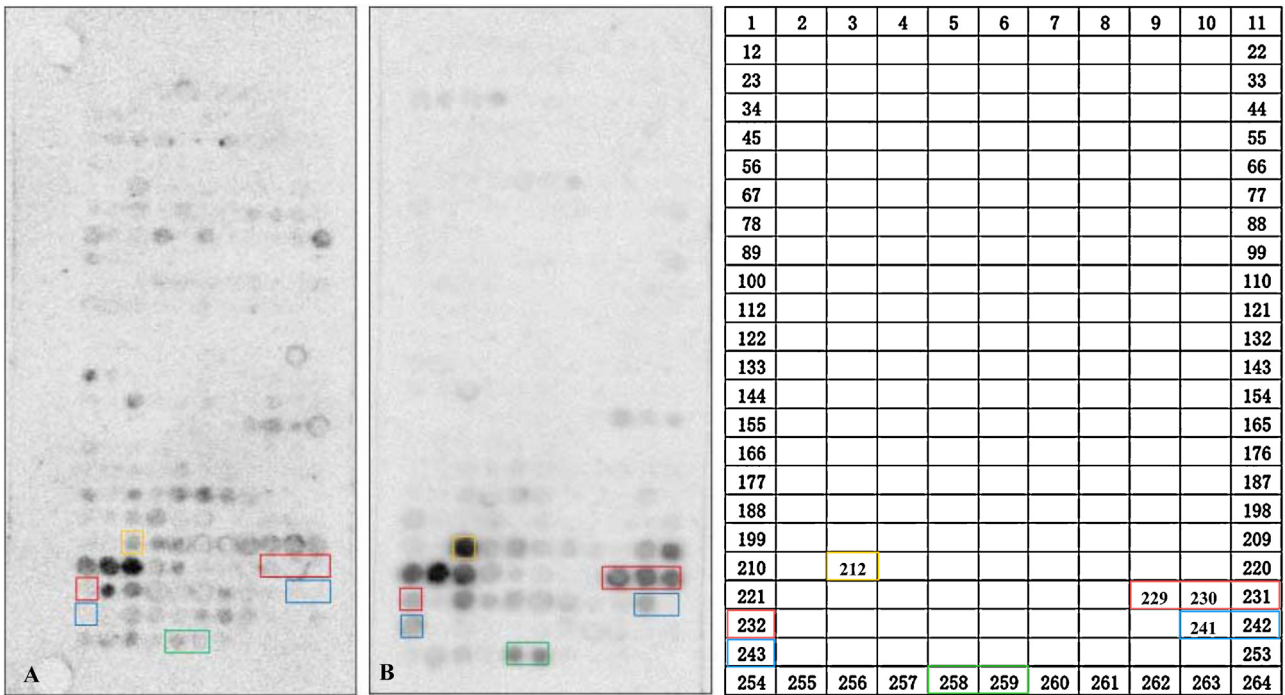


Fig. 2. Detection of antigenic sites from SeV NP protein overlapping peptide array membranes. Membrane A shows the reaction with SeV negative control serum. Membrane B shows the reaction with SeV positive control serum. Peptides at sites 212, 229–232, 241–243 and 258–259 show higher avidity with SeV positive control serum than with negative control serum in the membrane.

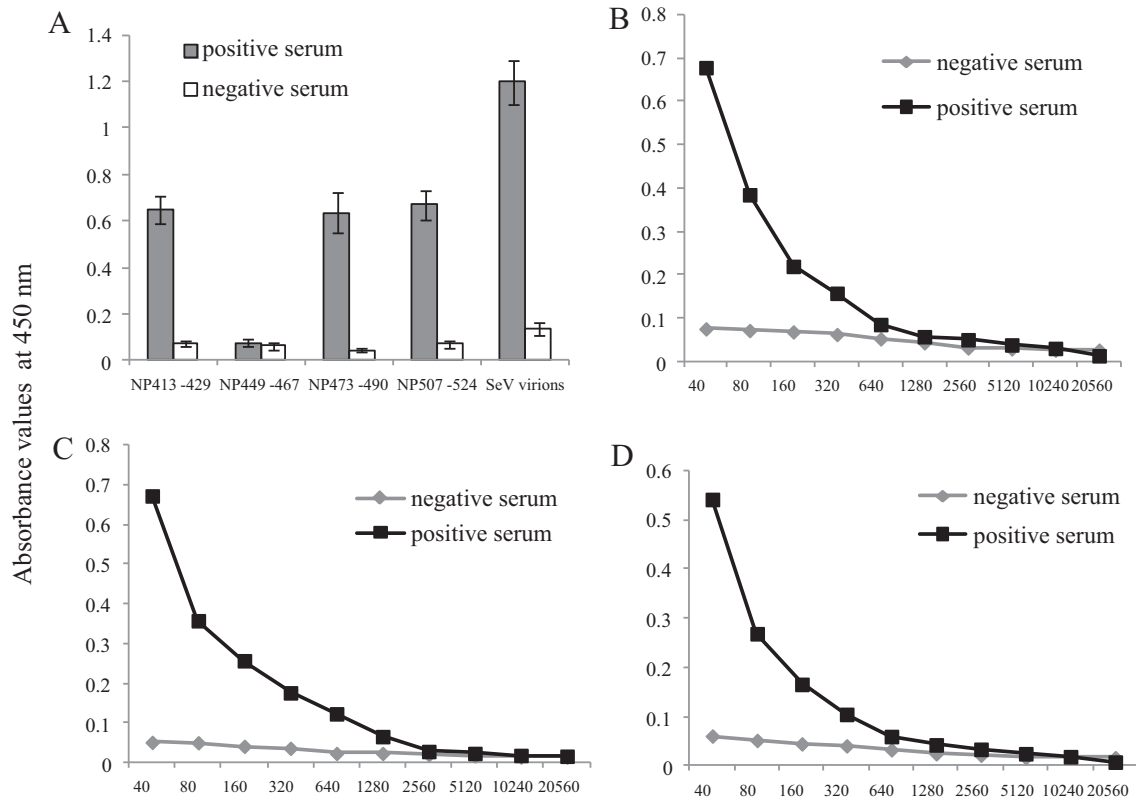


Fig. 3. ELISA of rat sera from peptide- and SeV complete virion-coated plates. (A) 1:40 dilution of positive and negative control sera were analyzed on peptide NP413–428-, NP449–467-, NP473–490-, NP507–524- and SeV complete virion-coated plates. (B–D) Sequentially diluted positive and negative control sera were analyzed on peptide NP413–428 (B), NP473–490 (C) and NP507–524 (D) coated plates.

Table 1
Peptides synthesized from SeV NP.

Peptide name	Peptide sequence	Position of amino acids
NP413–428	GGGAIEVALDNADIDL	413–428
NP449–468	WARSMGSGHFITLHGAERLE	449–468
NP473–490	DEDVSDIERRIARRLAER	473–490
NP507–524	VDHDEEDDAAAAAGMGGI	507–524

using these ELISA plates were performed (Fig. 3A). Negative control serum had no significant avidity on ELISA plates. Positive serum reactivity was more than five times higher than negative serum reactivity on peptide NP413–428-, NP473–490- and NP507–524-coated ELISA plates; although positive serum reactivity on peptide-coated ELISA plates was lower than complete virions. The specific avidity of SeV positive serum was not detectable on peptide NP449–468-coated plates. Sequentially diluted positive and negative control sera were also tested on peptide NP413–428-, NP473–490- and NP507–524-coated plates (Fig. 3B–D). Absorbance values significantly decreased as positive serum was diluted sequentially on all peptide-coated plates. Reaction values slightly decreased as negative serum dilution increased. All negative serum values were lower than 0.1 at OD450.

To explore the applicability of these peptide antigens, a mixture of peptide NP413–428, NP473–490 and NP507–524 was coated on the same wells of an ELISA plate. Seventeen clinical sera samples were tested using the peptide mixture-coated plate and a SeV virion-coated plate (Fig. 4). Absorbance values of samples 3, 4, 6 and 7 on the SeV virion-coated plate were higher than the cut-off value, which was set by negative serum on the SeV virion-coated plate. Absorbance values of these four samples from the peptide mixture-coated plate were also higher than the cut-off value. Four of the 17 samples were determined as being SeV antibody-positive by the complete virion- or the peptide mixture-coated ELISA plates. The results were consistent with those of the commercial ELISA kit.

Due to much higher epitope densities of the peptide-coated plate, the four SeV positive samples showed comparable absorbance of the peptide mixture-coated plate to that of the SeV virion-coated plate. The absorbance of the 13 SeV negative sera from the peptide mixture-coated plate (0.046 ± 0.031) was lower than the absorbance from the complete virion-coated plate (0.084 ± 0.051 , $p < 0.05$). The lower absorbance of SeV negative sera

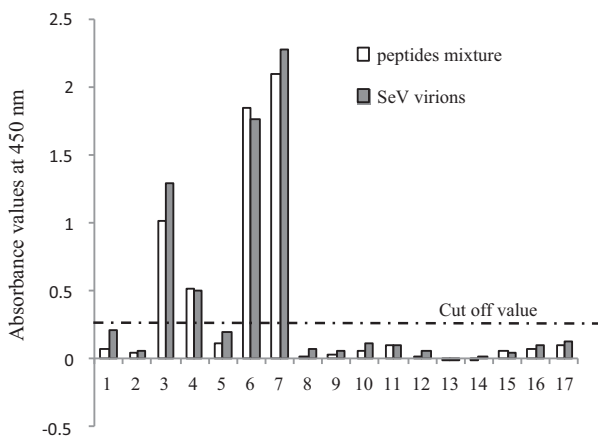


Fig. 4. ELISA results of 17 clinical rat sera reacted with peptide mixture- and SeV complete virion-coated plates. The absorbance values of sera samples have already omitted the absorbance value of the blank well. The cut-off value was set by the absorbance value of SeV negative control serum from SeV complete virion-coated plates +0.15. The absorbance values of samples 3, 4, 6 and 7 are higher than the cut-off value, both from the SeV complete virion-coated plate and the peptide mixture-coated plate.

from the peptide mixture-coated plate may imply better specificity of these peptides than SeV complete virions.

4. Discussion

The adventitious infection of laboratory animals by pathogens can lead to clinical disease and pathological changes. Even sub-clinical infections can lead to material biological changes which may impact on the results of experiments. SeV is one of the most important pathogens causing infection in this context, and should be monitored by routine surveillance (Schoondermark-van de Ven et al., 2006).

Serological methods, including ELISA, have been employed by different groups to detect anti-SeV antibodies (Ertl et al., 1979; Suzuki et al., 1987; Yanabe and Yoshida, 1986). The quality of antigens coated on plates is the most important factor affecting the accuracy of ELISA. Currently, SeV complete virions are used as ELISA antigens. Although tissue control had been introduced for this type of analysis to remove any unspecific binding influences, possible cross-reactions of SeV complete virions with other pathogens may lead to false positives (Ito et al., 1987).

In the current study, the predominant antigenic NP of SeV was screened by an overlapping peptides array and three antigenic peptides were identified, which were located in the carboxyl-terminus of NP.

The overlapping peptide array has previously been employed by different research groups to screen antigenic epitopes (Asano et al., 2011; Bluthner et al., 2000; Carter and Loomis-Price, 2004). In the current study, reactions of SeV positive serum and SeV negative serum with peptide membranes were compared, and four peptides were identified, which showed higher avidity with SeV positive serum. Some unexpected bindings were also detected. For example, SeV negative serum identified some peptides which were not identified by SeV positive serum (Fig. 2). The complexity of the sera and the unspecific binding may have contributed to this result.

To confirm the antigenicity of the four candidate peptides, peptides covering these four positions were synthesized and coated on ELISA plates. The antigenicity of three of the peptides was confirmed. Although overlapping peptides for amino acids 449–468 of NP had higher avidity with SeV positive serum, synthesized NP449–468 failed to show antigenicity in ELISA tests. NP449–468 also failed to show antigenicity in repeated experiments. These results may be due to the antigenicity displayed of synthesized NP449–468 on coated ELISA plates differing from the overlapping peptides synthesized directly on the membrane.

NP is a key structural unit which exists in many viruses, including Hantaan virus, coronaviruses, influenza viruses, and parainfluenza viruses (Elliott et al., 1984; Galinski et al., 1986; Kamata and Watanabe, 1977; Masters and Sturman, 1990). A SeV NP sequence comparative analysis was performed by DELTA-BLAST in a non-redundant protein sequences database (Boratyn et al., 2012). The homologous sequence of SeV NP is also found in NP from the following viruses: Human parainfluenza virus, Bovine parainfluenza virus, Simian Agent 10, Mossman virus, Cedar virus, Canine distemper virus, Nariva virus, Peste-des-petits-ruminants virus, Measles virus, Rinderpest virus and Tailam virus. The homologous sequence of SeV NP is located mainly at amino acids 1–350. In the current study, all three linear antigenic peptides found were located in the carboxyl-terminus of SeV NP. The NP carboxyl-terminal sequence of SeV is comparably unique to SeV NP. SeV negative rat sera samples had lower avidity with the three peptides than SeV complete virions (Figs. 3 and 4). Additionally, a mixture of these three peptides had no cross-reaction with mouse hepatitis virus, rat corona virus or sialodacryoadenitis virus, the most prevalent pathogens affecting mice and rats (data not shown). The results

suggest that the three peptides identified may be SeV-specific epitopes, and possibly have better specificity for SeV in serological detection.

In conclusion, this study identified three antigenic peptides with specificity for SeV. Primary clinical tests of 17 sera samples suggest that a mixture of these three peptides has comparable antigenicity and better specificity compared with SeV complete virions. Future tests using larger clinical samples will further elucidate the specificity and sensitivity of these peptide antigens.

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