A Small Region of Porcine Hemagglutinating Encephalomyelitis Virus Spike Protein Interacts with the Neural Cell Adhesion Molecule

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Key Words
Porcine hemagglutinating encephalomyelitis virus · Spike protein · Neural cell adhesion molecule

Abstract
Objective: The spike (S) protein of porcine hemagglutinating encephalomyelitis virus (PHEV) may mediate infection by binding to a cellular neural cell adhesion molecule (NCAM). This study aimed to identify the crucial domain of the S1 subunit of the S protein that interacts with NCAM. Methods: Three truncated segments (S1–291, S277–794, and S548–868) of the S gene of PHEV and the NCAM gene were cloned individually into the Escherichia coli expression vectors and yeast two-hybrid expression vectors. The interaction between S1–291, S277–794, S548–868, and NCAM were detected by a GST pull-down experiment and yeast two-hybrid assay. Results: Three fusion proteins (S1–291, S277–794, and S548–868) were screened for their interactions with NCAM by protein-protein interaction assays. The results of these assays clarified that S277–794 interacted with NCAM, while S1–291 and S548–868 did not. Conclusions: A small fragment (258-amino-acid fragment, residues 291–548) on the PHEV S protein was posited to be the minimum number of amino acids necessary to interact with NCAM. This fragment may be the receptor-binding domain that mediates PHEV binding to NCAM.

Introduction
Porcine hemagglutinating encephalomyelitis virus (PHEV) is a positive, nonsegmented, single-stranded RNA coronavirus belonging to the Betacoronavirus genus within the Coronaviridae family. Other related members are mouse hepatitis virus, bovine coronavirus, human coronavirus OC43, human coronavirus HKU1, etc. [1]. PHEV causes vomiting and wasting disease, as well as encephalomyelitis in piglets younger than 3 weeks, particularly in those lacking PHEV antibodies such as SPF pigs [2]. Recently, more research has focused on PHEV because infection rates have risen in some countries [2–6]. PHEV is a highly neurovirulent virus that spreads to the central nervous system via peripheral nerves, where nerve cells are a target for viral replication [7], and this spread could result in high neuropathogenicity. However, the mechanism by which PHEV enters the nerve cells remains largely unknown.

The coronavirus spike (S) protein is a key protein for determining the host and the tissue specificity of the virus, and plays an important role in coronavirus infection and pathogenicity [8–10]. The receptor of the coronaviral S protein is responsible for viruses entering into cells via...
fusion of viral envelope and the cellular membrane [11]. For example, the entry of SARS-CoV into cells is initiated by the binding of the S protein to the cellular receptor angiotensin-converting enzyme 2 [12], and the S protein of MERS-CoV targets the cellular receptor dipeptidyl peptidase 4 [13]. The neural cell adhesion molecule (NCAM, also known as CD56) is a homophilic glycoprotein expressed on the surface of nerve cells. NCAM has been implicated as having a role in cell-cell adhesion, neurite outgrowth and synaptic plasticity [14]. In 2010, Gao et al. [15] confirmed that NCAM participates in the process by which PHEV infects neurons. Therefore, NCAM may be a receptor for PHEV in N2a cells.

The coronaviral receptor-binding domain (RBD), which mediates the binding of viruses to receptors on susceptible cells, is the essential segment for triggering viral entry into target cells. A 193-amino-acid fragment of the SARS-CoV S protein has been identified as the RBD, which can efficiently bind angiotensin-converting enzyme 2 [16] and induce potent neutralizing antibodies and protection against SARS-CoV infection in an animal model [17]. Recently, a 286-amino-acid fragment (residues 377–662) of the MERS-CoV S protein that contains the viral RBD has also been successfully identified [18].

In a preliminary investigation, the coronaviral RBD of viruses such as SARS-CoV and MERS-CoV was found to be located on the S1 subunit of the S protein [12, 13]. To identify the crucial domain of the S1 subunit that interacts with NCAM, three truncated fusion proteins spanning the entire S1 subunit were prepared. These recombinant proteins were screened using a GST pull-down experiment, and the interactions were further confirmed by a yeast two-hybrid system assay. The results showed that the S277–794 fragment could interact with NCAM, and a smaller fragment (258-amino-acid fragment, residues 291–548) located in S277–794 may be the RBD of the PHEV S protein. These results will be the foundation for researching the mechanism of PHEV infection. In addition, recombinant proteins derived from the RBD could serve as targets for antiviral chemotherapy or a vaccine.

### Materials and Methods

**Viruses and Cells**

The HEV-67N strain of PHEV (ATCCVR741) [20] was conserved by the veterinary pathology laboratory of Jilin University and was propagated and assayed by the plaque method in N2a cell culture, as described previously [21].

**PCR Amplification and Construction of the Expression Vector**

According to the published standard strains of the HEV-67N gene sequence (Genbank accession No. AY078417), these primers were designed and synthesized to amplify the three truncated segments (fig. 1; table 1). The RT-PCR products were transferred into the expression vector pGEX-4T-1 (denoted pGEX S1–291, pGEX S277–794, pGEX S548–868).

| Gene Orientation | Sequence                                      |
|-------------------|-----------------------------------------------|
| S1–291 F          | 5′-ggatccATGTTTTTTATACCTTTTTAA-3′             |
| R                 | 5′-ctcgagTCATAAAATC ACTAGAC-3′                |
| S277–794 F        | 5′-ggatccGGTGT TTATACCCATGCTGT-3′            |
| R                 | 5′-ctcgagAATGA ACTCAAATTACC-3′               |
| S548–868 F        | 5′-ggatccGTTGG GCTTGTGGAG-3′                 |
| R                 | 5′-ctcgagCTTG ACTAAGGGTGAC-3′                |

Nucleotide sequences underlined are restriction sites.

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**Fig. 1.** Diagram of the expression constructs for the expression of soluble, truncated PHEV S glycoprotein.

**Table 1.** Sequence of primers used in this work

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**A Small Region of PHEV Spike Protein That Interacts with NCAM**

Intervirology 2015;58:130–137
DOI: 10.1159/000381060
The primers were designed according to Gao et al. [15] and the forward primer for the NCAM gene (5′-CGGAATTCGTGCCATCATTAAGCTCTGAAGT-3′) and the reverse primer for the S gene (5′-TTGCGGCCGCAAGTATGCCCTGGCCTGTAATG-3′) introduced BsmHI and EcoRI sites, respectively, using total RNA from the N2a cells as a template for PCR amplification. The PCR product was ligated into the expression vector pET28a (denoted as pET-NC).

Fusion Protein Expression

The plasmids pGEX-4T-1, pGEXS 1–291, pGEXS 277–794, pGEXS 548–868 and pET-NC were individually transformed in *Escherichia coli* BL21 cells separately. The GST alone, S 1–291-GST, S 277–794-GST and S 548–868-GST were purified with Glutathione Sepharose 4B beads (Amersham-Pharmacia Biotech). The NC-His fusion protein was purified using a His-Trap HP column. (Amersham Biosciences AB, Uppsala, Sweden). The identities of the proteins were analyzed by SDS-PAGE electrophoresis.

GST Pull-Down Assay

The Glutathione Sepharose 4B beads which adsorbed fusion protein S 1–291-GST, S 277–794-GST and S 548–868-GST were washed three times with PBS, and suspended the deposits with 200 μl of NETN buffer [50 mmol/l Tris-HCl (pH 7.5), 150 mmol/l NaCl, 1 mmol/l EDTA, 0.1 mmol/l DTT, 0.1% NP-40, 20% glycerol and 0.1% protease inhibitor]. The NC-His fusion protein was added to the solution and the purified GST protein, as a control, at 4° for more than 8 h. Then, the pellets of the Sepharose 4B beads were boiled in 80 μl of loading buffer, subjected to 15% SDS-PAGE, transferred to PVDF and blotted with His-specific antibodies.

Yeast Two-Hybrid Assay

Three gene segments containing the S1–291, S277–794 and S548–868 genes were excised from the plasmids pGEXS1–291, pGEXS277–794 and pGEXS548–868. The excision was blunt and was repaired with T4 DNA polymerase. The segments were ligated with the yeast two-hybrid expression vectors to create the plasmids pGADTS1–291, pGADTS277–794 and pGADTS548–868. Similarly, the NCAM gene was cloned into pGBK7 to create the plasmid pGBK7-NC. The yeast strain Y187 was transformed with the pGADTS1–291, pGADTS277–794 and pGADTS548–868 plasmids, respectively, and the yeast strain Y2HGold was transformed with the pGBK7-NC plasmid. The yeast Y187 containing pGADTS1–291, pGADTS277–794 or pGADTS548–868 was cultured on SD/-Leu, SD/-Leu/Xa-Gal, SD/-Leu/Tp and SD/-Leu/Xa-Gal/AbA solid plates, respectively, and the yeast Y2HGold containing pGBK7-NC was cultured on SD/-Trp, SD/-Trp/Xa-Gal, SD/-Trp and SD/-Trp/Xa-Gal/AbA solid mediums at 30° for 3–4 days. The strains containing S1–291, S277–794, S548–868 and the NCAM were monitored for autoactivation and toxicity to the yeast.

Each positive clone was chosen from Y187 containing pGADTS1–291, pGADTS277–794 and pGADTS548–868, or Y2HGold containing pGBK7-NC, and was cotransferred into a 1.5-ml centrifuge tube with 500 μl of 2× YPDA, mixed and stored at 30° for 20–24 h. The appropriate yeast were cultured onto SD/-Trp, SD/-Leu and SD/-Leu/Tp solid mediums and incubated at 30° for 3–5 days. Positive clones were chosen on the SD/-Leu/Tp solid mediums and were cultured onto SD/-Leu/Xa-Gal/AbA and SD/-Ade/-His/-Leu/-Trp/Xa-Gal/AbA solid medium, incubated at 30° for 3–5 days and examined for the growth of yeast strains.

Results

**PCR Amplification and Vector Construction**

According to the published standard strains of the HEV-67N gene sequence (Genbank accession No. AY078417), we designed and synthesized three sections, and the target genes were amplified by PCR amplification. These gene products were purified by gel extraction and were the expected sizes (fig. 2). Three recombinant vectors, pGEX S1–291, pGEX S277–794 and pGEX S548–868 were created by ligating the three products into the pEGX-4T-1 (fig. 3a).

Total RNA was extracted from the N2a cells and used as a template for PCR amplification. A resulting product...
of approximately 1,281 bp was obtained (fig. 2). A recombinant vector, pET-NC, was constructed by ligating this product with pET28a (fig. 3b).

**GST Pull-Down Experiment**

The recombinant plasmids pGEX S1–291, pGEX S277–794, pGEX S548–868, pGEX-4T-1 and pET-NC were transformed into *E. coli* BL21 cells. After isopropyl-β-D-thiogalactopyranoside induction, the fusion proteins (S1–291-GST, S277–794-GST, S548–868-GST and NC-His) were obtained (fig. 4). The results of the GST pull-down experiment showed that S277–794 could interact with NC-His, but there were no interactions between NCAM and S1–291, or S548–868 (fig. 5).

**Identification of Interaction by Yeast Two-Hybrid System**

The recombinant plasmids pGADT7 S1–291, pGADT7 S277–794 and pGADT7 S548–868 were transformed individually into yeast Y187. Simultaneously, the plasmid pGBK7T7NC was transformed into Y2HGold, and we observed no autoactivation (table 2). The pGADT7 S277–794 plasmid was transferred into the yeast Y187, noted as b1, and the pGBK7T7NC plasmid was transferred into the yeast Y2HGold, noted as NC (table 3). The transformed yeast b1 and NC only grew on SD/-Leu plates and SD/-Trp plates, respectively (tables 2, 3). The diploid yeast (pGADT7 S7–522 × pGBK7T7NC) is denoted as Y2, and the diploid yeast (pGBK7T7NC × pGADT7), (pGADT7

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**Fig. 3.** Analysis of PCR of recombinant plasmids. a Analysis of PCR of pGADT7 S1–291, lane 1 (873 bp); pGADT7 S548–868, lane 2 (960 bp), and pGADT7 S277–794, lane 3 (1,548 bp). Lane M, DNA marker DL-2000. b Analysis of PCR of pGBK7T7NC, lane 1 (1,281 bp). Lane M, DNA marker DL-2000.

**Fig. 4.** Fusion proteins of GST-S1–291, GST-S277–794, GST-S548–868, GST and NC-His were expressed and purified. a Fusion proteins of GST-S1–291 (58 kDa) were expressed and purified. b Fusion proteins of GST-S277–794 (84 kDa) were expressed and purified. c Fusion proteins of GST-S548–868 (61 kDa) were expressed and purified. d Fusion proteins of NC-His (48 kDa) were expressed and purified. e Fusion proteins of GST were expressed and purified.
S\textsubscript{277–794} \times pGBK7T), (pGBK7T-Lam \times pGADT7-T) and
(pGBK7T-53 \times pGADT7-T) are denoted as n, b2, N and
P (table 3). The results showed that Y2 grew blue colonies
on SD/-Trp/X-a-Gal/AbA plates and on SD/-Ade/-His/-Leu/-Trp/X-a-Gal/AbA plates (table 3; fig. 6). These
results suggested that S\textsubscript{277–794} could interact with NCAM.

Similarly, we also transferred pGADT7 S\textsubscript{1–291} and
pGADT7 S\textsubscript{548–868} separately into yeast Y1, denoted as
a1 and c1 (table 3). Y1 and Y3 failed in growth on
SD/-Ade/-His/-Leu/-Trp/X-a-Gal/AbA plates (table 3; fig. 6), indicating that S\textsubscript{277–794} could interact with NCAM
but S\textsubscript{1–291} and S\textsubscript{548–868} could not.

### Discussion

PHEV is a member of the Coronaviridae family. Com-
pared to other coronaviruses, the mechanism of its patho-
genesis and the characterization of its RBD are relatively
unknown. It is widely recognized that the main determi-
nant of coronavirus tropism is the viral S protein which
mediates binding to a cell surface receptor [22]. The S pro-
teins of coronaviruses have an S1 and an S2 subunit. The S1
subunit initiates entry of viruses into cells by binding to cell
surface receptors, and the S2 subunit helps viral fusion with
cellular membranes [23–25]. The RBDs present in the S1
subunit is important for interaction with receptors. Wong
et al. [16] reported that a 193-amino-acid fragment of the
SARS-CoV S protein (residues 318–510) has been shown
to interact with angiotensin-converting enzyme 2, and re-
cently, Mou et al. [22] found that a 231-amino-acid frag-
ment of the MERS-CoV S protein (residues 358–588) could
interact with the cellular receptor dipeptidyl peptidase 4.
Therefore, the S1 subunits of S proteins are crucial for the
interactions of coronaviruses with specific receptors.

The PHEV S protein is composed of 1,349 amino ac-
cids. Based on bioinformatics analysis and the S proteins
of other coronaviruses, there is a transmembrane helix
between the 1,294 amino acid and 1,316 amino acid in the
PHEV S protein. Residues 1–1,293 are located in the extracel-
llular region, and residues 1,317–1,349 are intracel-
ular. Residues 1–794 of the PHEV S protein compose the

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**Table 2. Autoactivation results of the transformed yeast strains**

| Yeast strain (plasmid) | Selective agar plate | Colony | Color |
|------------------------|----------------------|--------|-------|
| Y187(pGADT7S\textsubscript{1–291})/Y187(pGADT7S\textsubscript{277–794})/Y187(pGADT7S\textsubscript{548–868}) | SD/-Leu | Yes | White |
| | SD/-Leu/X-a-Gal | Yes | White |
| | SD/-Leu/-Trp | No | No |
| | SD/-Leu/X-a-Gal/AbA | No | No |
| Y2Hgold(pGBK7TNC) | SD/-Trp | Yes | White |
| | SD/-Trp/X-a-Gal | Yes | White |
| | SD/-Leu/-Trp | No | No |
| | SD/-Trp/X-a-Gal/AbA | No | No |
| Y2HGold(pGBK7T-53)\times Y187(pGADT7-T) | SD/-Leu/-Trp | Yes | White |
| | SD/-Leu/-Trp/X-a-Gal/AbA | Yes | Blue |
| Y2HGold(pGBK7T-Lam)\times Y187(pGADT7-T) | SD/-Leu/-Trp | Yes | White |
| | SD/-Leu/-Trp/X-a-Gal/AbA | No | No |
Table 3. Analysis of the interactions between S protein and NCAM in yeast

| No. | Yeast plasmid | Selective agar plate | Colony | Color |
|-----|---------------|----------------------|--------|-------|
| a1, b1, c1 | Y187(pGADT7S1–291), Y187(pGADT7S277–794), Y187(pGADT7S548–868) | SD/-Leu | Yes | White |
| | Y187(pGADT7S548–868) | SD/-Leu/-Trp | No | No |
| NC | Y2Hgold(pGBK7T7NC) | SD/-Trp | Yes | White |
| | | SD/-Leu/-Trp | No | No |
| P | Y2Hgold(pGBK7T7-53)×Y187(pGADT7-T) | SD/-Leu/-Trp | Yes | White |
| | | SD/-Ade/-His/-Leu/-Trp | Yes | White |
| | | SD/-Ade/-His/-Leu/-Trp/X-a-Gal/AbA | Yes | Blue |
| N | Y2Hgold(pGBK7T7-Lam)×Y187(pGADT7-T) | SD/-Leu/-Trp | Yes | White |
| | | SD/-Leu/-Trp/X-a-Gal/AbA | No | No |
| n | Y2Hgold(pGBK7T7NC)×Y187(pGADT7T) | SD/-Leu/-Trp | Yes | White |
| | | SD/-Leu/-Trp/X-a-Gal/AbA | No | No |
| a2, b2, c2 | Y2Hgold(pGBK7T7)×Y187(pGADT7S1–291), Y2Hgold(pGBK7T7)×Y187(pGADT7S277–794), Y2Hgold(pGBK7T7)×Y187(pGADT7S548–868) | SD/-Leu/-Trp | Yes | White |
| | | SD/-Leu/-Trp/X-a-Gal/AbA | No | No |
| Y1 | Y2Hgold(pGBK7T7NC)×Y187(pGADT7S1–291) | SD/-Leu/-Trp | Yes | White |
| | | SD/-Ade/-His/-Leu/-Trp | No | No |
| | | SD/-Ade/-His/-Leu/-Trp/X-a-Gal/AbA | No | No |
| Y2 | Y2Hgold(pGBK7T7NC)×Y187(pGADT7S277–794) | SD/-Leu/-Trp | Yes | White |
| | | SD/-Ade/-His/-Leu/-Trp | Yes | White |
| | | SD/-Ade/-His/-Leu/-Trp/X-a-Gal/AbA | Yes | Blue |
| Y3 | Y2Hgold(pGBK7T7NC)×Y187(pGADT7S548–868) | SD/-Leu/-Trp | Yes | White |
| | | SD/-Ade/-His/-Leu/-Trp | No | No |
| | | SD/-Ade/-His/-Leu/-Trp/X-a-Gal/AbA | No | No |
S1 subunit, and residues 795–1,349 compose the S2 subunit. Although it has been reported that the PHEV S protein interacts with NCAM [15], the crucial binding domain of the PHEV S protein is unknown. Therefore, in this study, we prepared three truncated fusion proteins spanning the entire S1 subunit of the PHEV S protein to identify the crucial domain of interaction with NCAM.

In our work, we used the GST pull-down assay to elucidate the interaction between the PHEV S protein segments and NCAM. The NCAM that was generated using pET28a with a His-tag formed a stable complex with the GST-S1–291, GST-S277–794 and GST-S548–868. The results showed that the fragment S277–794 interacted with NCAM. Additionally, these results are supported by the yeast two-hybrid system assay. The yeast two-hybrid study showed that the fragments S1–291, S277–794 and S548–868 are not autoactivating (table 2). The diploid yeast Y1, Y2, Y3, N and P, which were coated on SD/-Leu/-Trp plates, respectively, could grow white colonies (table 3), indicating that these plasmids were successfully transferred to the diploid yeast. The white colonies were inoculated separately on SD/-Leu/-Trp/Xa-Gal/AbA plates. However, only the diploid yeast Y2 and the positive control P grew colonies, which were blue (table 3). The diploid yeast Y2 could also grow colonies on an SD/-Ade/-His/-Leu/-Trp/Xa-Gal/AbA plate, and these colonies were also blue (table 3; fig. 6). These findings further demonstrate that the amino acid fragment S277–794 may specifically interact with NCAM; however, there was no interaction between NCAM and the fragments S1–291, or S548–868.

Based on these results, we found an association between NCAM and S277–794; however, S1–291 and S548–868 showed no such association. S277–794 is located in the S1 subunit. In addition, because S1–291 and S548–868 could not interact with NCAM, and there are overlapping regions between S277–794 and S1–291 as well as S277–794 and S548–868, we conclude that the S1 subunit may contain a cellular receptor-binding region up to 258 amino acids long. These findings suggested that the 258-amino-acid fragment, residues 291–548, may be the NCAM RBD of the S protein. This region may mediate PHEV binding to NCAM, but further studies are needed to confirm these results. Meanwhile, S1–291 and S548–868 did not interact with NCAM and might be less relevant to bind the cellular receptor.

These amino acid residues should be further investigated to further identify the potentially crucial amino acids that interact between the S protein and NCAM. In addition, these findings may contribute in identifying the effective target epitope for an S protein-based subunit vaccine.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (Nos. 31472194, 31272530, 31201875 and 31172291), the Program for Changjiang Scholars and Innovative Research Teams in University (PCSIRT, No. IRT1248) and the National Science Foundation of Jilin Province (Nos. 20130101106JC and 201115040).

References

1. Masters PS: The molecular biology of coronaviruses. Adv Virus Res 2006;66:193–292.
2. Quiroga MA, Cappuccio J, Pineyro P, Basso W, More G, Kienast M, Schonfeld S, Cancer JL, Arauz S, Pintos ME, Nanni M, Machuca M, Hirano N, Perfumo CJ: Hemagglutinating encephalomyelitis coronavirus infection in pigs, Argentina. Emerg Infect Dis 2008;14:484–486.
3. Mengeling WL, Boothe AD, Ritchie AE: Characteristics of a coronavirus (strain 67N) of pigs. Am J Vet Res 1972;33:297–308.
4. Cartwright SF, Lucas M, Huck RA: A small haemagglutinating porcine DNA virus. I. Isolation and properties. J Comp Pathol 1969;79:371–377.
5. Gao W, Zhao K, Zhao C, Du C, Ren W, Song D, Lu H, Chen K, Li Z, Lan Y, Xie S, He W, Gao F: Vomiting and wasting disease associated with hemagglutinating encephalomyelitis viruses infection in piglets in Jilin, China. Virol J 2011;8:130.
6. Rho S, Moon HJ, Park SJ, Kim HK, Keum HO, Han JY, Van Nguyen G, Park BK: Detection and genetic analysis of porcine hemagglutinating encephalomyelitis virus in South Korea. Virus Genes 2011;42:90–96.
7. Hara Y, Hasebe R, Sunden Y, Ochiai K, Honda E, Sakoda Y, Umemura T: Propagation of swine hemagglutinating encephalomyelitis virus and pseudorabies virus in dorsal root ganglia cells. J Vet Med Sci 2009;71:595–601.
8. Iacono KT, Kazi I, Weiss SR: Both spike and background genes contribute to murine coronavirus neurovirulence. J Virol 2006;80:6834–6843.
9. Li WH, Moore MJ, Vasileva N, Sui JH, Wong SK, Berne MA, Somasundaran M, Sullivan JL, Luzuriaga K, Greenough TC: Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature 2003;426:450–454.
10. Miura TA, Travanty EA, Oko L, Bielefeldt-Ohmann H, Weiss SR, Beauchemin N, Holmes KV: The spike glycoprotein of murine coronavirus MHV-JHM mediates receptor-independent infection and spread in the central nervous systems of Ceacam1a−/− mice. J Virol 2008;82:755–763.
11. Liu S, Xiao G, Chen Y, He Y, Niu J, Escalante CR, Xiong H, Farnar D, Debnath AK, Tien P, Jiang S: Interaction between heptad repeat 1 and 2 regions in spike protein of SARS-associated coronavirus: implications for virus fusogenic mechanism and identification of fusion inhibitors. Lancet 2004;363:938–947.
12. Li W, Moore MJ, Vasileva N, Sui J, Wong SK, Berne MA, Somasundaran M, Sullivan JL, Luzuriaga K, Greenough TC, Choe H, Farzan M: Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature 2003;426:450–454.
Raj VS, Mou H, Smits SL, Dekkers DH, Müller MA, Dijkman R, Muth D, Demmers JA, Zaki A, Fouchier RA, Thiel V, Drosten C, Rottier PJ, Osterhaus AD, Bosch BJ, Haagmans BL: Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. Nature 2013;495:251–254.

Auer S, Lappalainen RS, Skottman H, Suuronen R, Narkilahti S, Vikholm-Lundin I: An antibody surface for selective neuronal cell attachment. J Neurosci Methods 2010;186:72–76.

Gao W, He W, Zhao K, Lu H, Ren W, Du C, Chen K, Lan Y, Song D, Gao F: Identification of NCAM that interacts with the PHE-CoV spike protein. Virol J 2010;7:254.

Wong SK, Li W, Moore MJ, Choe H, Farzan M: A 193-amino acid fragment of the SARS coronavirus S protein efficiently binds angiotensin-converting enzyme 2. J Biol Chem 2004;279:3197–3201.

Du L, He Y, Zhou Y, Liu S, Zheng BJ, Jiang S: The spike protein of SARS-CoV: a target for vaccine and therapeutic development. Nat Rev Microbiol 2009;7:226–236.

Du L, Zhao G, Kou Z, Ma C, Sun S, Poon VK, Lu L, Wang L, Debnath AK, Zheng BJ, Zhou Y, Jiang S: Identification of a receptor-binding domain in the S protein of the novel human coronavirus Middle East respiratory syndrome coronavirus as an essential target for vaccine development. J Virol 2013;87:9939–9942.

Zhu X, Liu Q, Du L, Lu L, Jiang S: Receptor-binding domain as a target for developing SARS vaccines. J Thorac Dis 2013;5(suppl 2):S142–S148.

Mengeling WL, Boothe AD, Ritchie AE: Characteristics of a coronavirus (strain 67N) of pigs. Am J Vet Res 1972;33:297–308.

Hirano N, Ono K, Takasawa H, Murakami T, Haga S: Replication and plaque formation of swine hemagglutinating encephalomyelitis virus (67N) in swine cell line, SK-K culture. J Virol Methods 1990;27:91–100.

Mou H, Raj VS, van Kuppeveld FJ, Rottier PJ, Haagmans BL, Bosch BJ: The receptor binding domain of the new Middle East respiratory syndrome coronavirus maps to a 231-residue region in the spike protein that efficiently elicits neutralizing antibodies. J Virol 2013;87:9379–9383.

Bonavia A, Zelus BD, Wentworth DE, Talbot PJ, Holmes KV: Identification of a receptor-binding domain of the spike glycoprotein of human coronavirus HCoV-229E. J Virol 2003;77:2530–2538.

Bresee JJ, Mork I, Smith MK, Vogel LK, Hemmila EM, Bonavia A, Talbot PJ, Sjöström H, Norén O, Holmes KV: Human coronavirus 229E: receptor binding domain and neutralization by soluble receptor degrees at 37°C. J Virol 2003;77:4435–4438.

Kubo H, Yamada YM, Taguchi F: Localization of neutralizing epitopes and the receptor-binding site within the amino-terminal 330 amino acids of the murine coronavirus spike protein. J Virol 1994;68:5403–5410.