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Phages bearing affinity peptides to severe acute respiratory syndromes-associated coronavirus differentiate this virus from other viruses

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\textbf{Abstract}

\textbf{Background:} Transmission of SARS-associated coronavirus (SARS-CoV) is now well controlled; nevertheless, it is important to develop effective methods to identify this virus from other pathogens.

\textbf{Objectives:} The purpose of this study was to identify potential ligands and develop a novel diagnostic test to SARS-CoV using phage display technology.

\textbf{Study design:} The SARS-CoV spike 1 (S1) protein containing the receptor binding region (RBD) was used as an immobilized target followed by incubation with a 12-mer phage display random peptide library. After four rounds of biopanning, 10 monoclonal phages with specific binding activity to the S1-RBD protein were obtained and subjected to binding and diagnostic assays.

\textbf{Results:} DNA sequencing showed that two phage displayed peptides HHTWHPPVMHL (phage-H) and SQWHPRSASYPM (phage-S) that were specific ligands to the S1 protein. Moreover, the selected phage-H and phage-S were capable of differentiating SARS-CoV from other coronaviruses in indirect enzyme-linked immunosorbent assays.

\textbf{Conclusion:} The peptides identified in this study are useful reagents for detection of SARS-CoV.

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\section{1. Background}

Severe acute respiratory syndrome (SARS) caused by SARS-associated coronavirus (SARS-CoV) was a life-threatening disease and widespread in 2003. Although transmission of SARS-CoV has been well controlled currently, it is necessary to develop effective methods to identify this virus from other pathogens.

SARS-CoV is an enveloped, single-stranded positive-sense RNA virus with a genome that is about 30,000 nucleotides in length and encodes at least 15 open reading frames.\textsuperscript{1,2} SARS-CoV encodes four major structural proteins: spike (S) protein, nucleocapsid (N) protein, membrane (M) protein and small envelope (E) protein.\textsuperscript{1,2} Cell infection by SARS-CoV is initiated by the interaction of the surface S protein with human angiotensin-converting enzyme 2 (hACE2).\textsuperscript{3,−5} The hACE2 protein was identified as a functional receptor for the SARS-CoV and its binding site on the S protein was localized between amino acids 318 and 510.\textsuperscript{3,−5}

\section{2. Objectives}

One purpose of this study was to identify potential ligands to SARS-CoV by the biopanning assay of SARS-CoV S1 protein with a phage display random library. Another purpose is to develop a phage-based ELISA to distinguish SARS-CoV from other control viruses.

\section{3. Study design}

\subsection{3.1. Virus and other reagents}

Transmissible gastroenteritis (TGEV) isolate HR/DN1, porcine epidemic diarrhea virus (PEDV) HLJBY,\textsuperscript{17} porcine reproductive and
respiratory syndrome virus (PRRSV) isolate HH08\(^{18}\) and infectious bronchitis virus (IBV) strain HH06,\(^{19}\) human coronavirus 229E (HCoV 229E)\(^{20}\) and murine hepatitis virus strain A59 (MHV-A59)\(^{21}\) were kept in our laboratory. The SARS-CoV strain BJ-01 was a generous gift from Dr. Chengfeng Qin, Beijing Institute of Microbiology and Epidemiology, China. For virus inactivation, the SARS-CoV was treated with \(\beta\)-propiolactone followed by concentration with PEG20000 and purification by Sepharose 4F column chromatography as detailed in a previous report.\(^{22}\) Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-mouse IgG were purchased from the Zhongshan Company (Beijing, China).

3.2. Expression and purification of S1 protein in Escherichia coli

Expression of SARS-CoV S1 protein was performed as described previously.\(^{23}\) A recombinant plasmid bearing full-length SARS-CoV S gene (GenBank accession number AY278554) was used as PCR template. Sense primer (CoVS1) \(5'-GGGGGgattATGGTTTTTACAC\) TTTG and antisense primer (CoVS2) \(5'−\)CCCGGagatCTTGGTAAA TGGTTGAAA were synthesized to amplify a truncated S gene (nucleotides 664-1656). After inserting the S1 gene into BamHI and EcoRI sites of pGEX-6p-1 prokaryotic expression vector (Amersham Biosciences, New York, NY), the recombinant plasmid was sequenced and designed as pGEX-SARS-S1. The pGEX-SARS-S1 plasmid was transformed into host cells E. coli BL21(DE3). Expression of target protein was induced using 0.5 mM isopropyl \(\beta\)-D-thiogalactoside (IPTG) at 37 °C. Subsequent purification of inclusion bodies and renaturation of fusion protein by dialysis were performed as previously described.\(^{23,24}\) The target protein was designated as S1 protein.

3.3. Biopanning and enrichment analysis

Phage display was performed using a Ph.D.-12 Phage Display Peptide Library Kit (EB1100) according to the manufacturer's instructions (New England Biolabs) with minor modifications. For the first round of panning, 96-well plates were coated with the purified S1 protein at a concentration of 15 μg/well in 0.1 M NaHCO\(_3\) (pH 8.6) buffer overnight at 4 °C. Then, these plates were blocked for 1 h at 4 °C with 5% skimmed milk diluted in 0.05% (v/v) Tween-20 in phosphate-buffered saline (PBST). Following six washes with TBST (50 mMTris–HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween-20), the S1 protein was incubated with the phage library at a final concentration of \(1.5 \times 10^{11}\) pfu (100 μL/well) at room temperature for 30 min with gentle rocking. The unbound washes were 10 times with TBST and the bound phages were eluted by adding 100 μL elution buffer (0.2 M glycine–HCl [pH 2.2]) at room temperature for 30 min. The elute neutralized with 15 μL of 1 M Tris–HCl (pH 9.1) was harvested followed by amplification and titration in E. coli ER2738.

The second, third and fourth rounds of panning were done by similar panning processes with the exception of gradually increased concentration of Tween-20 (0.5%, v/v) in TBST. In between each round of panning, the titer of the amplified phages in washing buffer (here referred to as Output) was determined, and their ratio was analyzed to evaluate the enrichment efficiency.

3.4. Binding analysis of individual phage using ELISA

Ten phage clones were subjected to ELISA. Briefly, ELISA plates were coated with the S1 protein diluted in 0.1 M NaHCO\(_3\) (pH 8.6) (10 μg/well) overnight at 4 °C. The mixed phages from the phage display library were used as a control. The second day, the plates were blocked with 1% TBSB for 2 h at room temperature. These plates were washed six times with TBST and then incubated with the selected monoclonal phage at a concentration of \(1.5 \times 10^{11}\) pfu/100 μL in 0.1 M NaHCO\(_3\) (pH 8.6) for 1 h at 37 °C. After six washes with TBST, rabbit anti-M13 polyclonal antibody (diluted 1:1000 in TBSS; Abcam) was added to these wells for another 1 h at 37 °C. After six washes with TBST, these wells were incubated with HRP-conjugated anti-rabbit IgG antibody (diluted 1:1000 in TBSS). The color was developed using o-phenylenediamine (OPD), and the optical density (OD) value was read using an ELISA reader at a wavelength of 490 nm.

3.5. PCR amplifying genes encoding exogenous phage-displayed peptides

The ten positive phage clones were amplified and precipitated with polyethylene glycol–NaCl. Each phage clone DNA was purified using a plasmid extraction kit (Qiagen, Germany). The purified DNA template and the primers +130M13 (5′-TCACCTC AAAAGCAAGGTG) and −28M13 (5′-CCCTCATAGTACCTGAAC) were used to PCR amplify the gene encoding the exogenous peptides of the M13 phages. DNA sequencing was carried out by the Borun Shanghai Company. The deduced amino acid sequences were analyzed using the Lasergene MegAlign software program (Lasergene 7, DNASTAR, Inc., USA).

3.6. Binding of phage-displayed peptide to transiently expressed SARS-CoV S1 protein

Binding of the identified phage-displayed peptides to the S1 protein was investigated. Recombinant plasmids bearing SARS-CoV S1 gene, TGEV S1 gene or PEDV S1 gene were constructed by inserting the respective gene into the pVAX-N1 vector resulting in pVAX-SARS-S1, pVAX-TGEV-S1 and pVAX-PEDV-S1, respectively. These plasmids were transfected into BHK 21 cells in 24-well plates using lipofectamine 2000™ transfection reagent (Invitrogen, USA) according to the manufacturer's instructions. At 24 h post-transfection, indirect immunofluorescence assays were performed with modifications as described.\(^{23,24}\) Cells fixed with 4% paraformaldehyde (w/v in PBS) were incubated with the identified phages and an unrelated phage bearing a control peptide (phage-control) \((1.5 \times 10^{11}\) pfu, diluted in PBS) for 1 h. After washing with PBS, the cells were incubated with the anti-M13 antibody (1:300 dilution in 1% BSA) followed by incubation with FITC-labeled goat anti-rabbit IgG (1:500 dilution in 1% BSA) for 1 h in the dark. After three washes with PBS, green fluorescence signals were analyzed by fluorescence microscope (Leica, Germany). The transient expression levels of the constructs were measured by selecting the central areas of the transfected cells (five wells/sample) to capture the fluorescence picture and the average fluorescence densities were determined with ImageJ software (developed at the National Institutes of Health).

3.7. Establishment of phage-mediated ELISA for virus diagnosis

The selected phages and phage library were used as diagnostic reagents to detect a panel of viruses composed of SARS-CoV, HCoV 229E, MHV-A59, TGEV, PEDV, PRRSV, and IBV; 0.1 M NaHCO\(_3\) (pH 8.6) was used as negative control. All the viruses of the same titer \((10^{6}\) pfu/mL) were diluted in 0.1 M NaHCO\(_3\) (pH 8.6) to a final concentration of 10 μg/well and coated onto ELISA plates overnight at 4 °C followed by ELISA analysis as above. The OD\(_{490}\) values were determined. At least three independent experiments were repeated. Statistical significance was evaluated using the t-test. The \(p < 0.01\) was considered highly significant statistically.
Table 1
Efficacy of panning to SARS-CoV S1 protein.

| Buffer of ratio | Titration value for round |
|-----------------|---------------------------|
|                 | First                     | Second                    | Third                     | Fourth                    |
| Input           | $1.5 \times 10^{11}$      | $1.5 \times 10^{11}$      | $1.5 \times 10^{11}$      | $1.5 \times 10^{11}$     |
| Output          | $5.3 \times 10^{11}$      | $5.3 \times 10^{11}$      | $3.5 \times 10^{11}$      | $1.2 \times 10^{11}$     |
| Amplifying      | $4.3 \times 10^{12}$      | $7.2 \times 10^{12}$      | $8.6 \times 10^{12}$      | $1.6 \times 10^{12}$     |
| Amplifying/Output| $8.1 \times 10^{9}$      | $1.4 \times 10^{9}$      | $2.5 \times 10^{9}$      | $1.3 \times 10^{9}$     |
| Output/Input    | $3.5 \times 10^{-8}$      | $3.5 \times 10^{-8}$      | $2.3 \times 10^{-8}$      | $0.8 \times 10^{-8}$     |

* The titration values (per mL) for phage in input buffer (Input), elution buffer (Output), and last washing buffer (Amplifying) and ratios of Amplifying/Output and Output/Input values in each round of panning are provided.

4. Results

4.1. Expression and purification of SARS-CoV S1 protein

The S1 gene of 1023 bp was amplified by PCR, cloned into pGEX-6p-1 vector and expressed in E. coli, resulting in a chimeric SARS-CoV S1 protein connected with the GST tag protein with a molecular weight of 64 kDa (38 kDa S1 protein plus 26 kDa GST-Tag protein). The expression and purification of the S1 protein have been reported recently.23

4.2. Biopanning to identify phages bearing specific peptides to SARS-CoV S1 protein

Four rounds of phage-based biopanning were performed using the expressed SARS-CoV S1 protein as a target. The enrichment efficacies of the phages in each round were compared. As shown in Table 1, the Output/Input value increased in the second round but decreased subsequently; the Amplifying/Output increased with increasing biopanning rounds. The binding activities of 10 selected phages were assayed using ELISA. The results revealed that they had a specific binding activity to the SARS-CoV S1 protein (Fig. 1).

4.3. Peptide sequences displayed on the phages to SARS-CoV S1 protein

DNA sequencing indicated that among the ten selected phages, 2 deduced peptide sequences (12 amino acids in length) were identified (Table 2), in which eight phages shared a consensus sequence and the other two phages shared another consensus sequence. Phages bearing peptides HHKTWHPVVMHL and SQWHPRSASYPM were named phages H and S, respectively. In addition, a putative motif, WHP, was determined using DNASTar software.

4.4. Phage-displayed peptides recognized transiently expressed SARS-CoV S1 protein

After transient expression of the S1 protein of SARS-CoV, TGEV or PEDV, their expression levels were determined with ImageJ software. As shown in Supplementary Fig. 1, the protein expression levels of the S1-SARS, TGEV-S1 and PEDV-S1 were similar. Then, the phages bearing either S1-specific peptides or phage library peptides were used to detect S1 proteins on the cell surface. As shown in Fig. 2, green fluorescence signals were detected only if pVAX-SARS-S1 was transfected into BHK-21 cells; in contrast, the phages did not react with TGEV or PEDV S1 proteins. At the same time, there was no unspecific reaction between the proteins and the control phage.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jcv.2013.04.002.

4.5. Phages expressing specific affinity peptides to S1 protein differentiated SARS-CoV from other viruses

Phage-H and phage-S were analyzed for their specificities in recognizing SARS-CoV and other viral agents. As shown in Fig. 3, the two selected phages revealed a significantly higher reactivity to inactivated SARS-CoV than to the control pathogens (p<0.01). Both selected phages showed a similar high reactivity to SARS-CoV.

5. Discussion

Previous work has demonstrated various utilities of the SARS-CoV S1 protein.1,5,23,26,27 Compared with other kinds of available expression systems, E. coli has advantages in protein yield, production cost, operation convenience, etc.28 In our previous study, the high-level expression of the SARS-CoV S1 protein in E. coli provided the important material for development of a novel detection method for SARS-CoV.23 Phage display has been used to isolate specific peptides to isolated proteins, inorganic material and complex target structures.29 In this study, the immobilized S1 protein was used as target. Several phages harboring different peptides were identified in protein-based ELISA for the first time. DNA sequencing indicated that 8 of the 10 selected phages shared a consensus peptide, and the other 2 shared a consensus peptide. Interestingly, a putative motif, WHP,

Table 2
Deduced amino acid sequences of phage-displayed peptides

| Clone ID | Sequence |
|----------|----------|
| Phages 1–5 and 8–10 | HHKTWHPVVMHL |
| Phages 6 and 7 | SQWHPRSASYPM |

* Ten selected phages (phages 1–10) were subjected to DNA extraction and PCR. The deduced amino acid sequences are shown. Underline showed putative motifs binding the SARS-CoV S1 protein.

Fig. 1. Binding analysis of the selected phages to SARS-CoV S1 protein in ELISA. Ten selected phages, phage 1 to phage 10, were incubated with the SARS-CoV S1 protein in ELISA plates to determine the specific binding activities for the protein as described in Study design. The OD (x axis) of tested individual phage and the control is shown on the y axis; the individual phage and the control phage complex from the phage library are shown on the x axis. Three independent experiments were repeated for each individual.

* Three independent experiments were repeated.
was found in all the 10 peptides, suggesting the selected motif may be indispensable in binding to SARS-CoV S1 protein. To confirm the specific recognition of the phage-displayed peptides to the S1 protein, we analyzed their interaction with the S1 proteins from several coronaviruses. The data showed that these identified phages recognized SARS-CoV S1 protein exclusively. They did not react with other S1 proteins indicating that the reaction is specific. At the same time, when we used the same phage bearing an unrelated peptide, no positive result was observed, implying that the reaction was mediated by the S1 protein specific peptides. It has been reported that little evolution has been occurring within SARS-CoV S proteins; however, more SARS-CoV isolates should be analyzed in the future to confirm the specificity of the phage-based ELISA. Interestingly, when we used the SARS-CoV S1 protein as sample in a Western blot, the phage displayed peptides did not recognize this protein, and this result was consistent with that of our ELISA with the use of denatured SARS-CoV S1 protein (data not shown). These pieces of evidence indicate that denaturation of the target protein may affect the interaction with their ligand peptides. Mutation of these identified peptide sequences will be done to clarify their function in our further study. In this study, we wanted to use the S1-binding peptides to establish an indirect ELISA to detect SARS-CoV. To analyze the specificity of such an ELISA, several viruses including MHV, a virus that is genetically more closely related to SARS-CoV, human coronavirus 229E, porcine coronaviruses TGEV and PEDV, the avian coronavirus IBV

![Fig. 2. Binding specificity of phage displayed peptides to SARS-CoV S1 protein. BHK 21 cells were transfected with pVAX-N1 vector, pVAX-SARS-S1, pVAX-TGEV-S1 and pVAX-PEDV-S1, respectively. At 24 h post-transfection, the fixed cells were incubated with the identified phages and an unrelated phage bearing a control peptide (phage-control) at a titer of $1.5 \times 10^{11}$ pfu followed by incubation with rabbit anti-M13 polyclonal antibody and FITC-labeled goat anti-rabbit IgG, respectively. A representative comparison is provided. (For interpretation of the references to color in text, the reader is referred to the web version of the article.)](image-url)
Two phages harboring specific peptides recognizing inactivated SARS-CoV were identified from the 10 selected phages. Two identified peptides were sequencing and designed as phage-H and phage-S, which were incubated with various kinds of virus in ELISA plates. \( \* p < 0.01 \) (highly significant) compared with HCoV-229E, MHV-A59, TGEV, PEDV, IBV and Coating buffer (NaHCO\(_3\)).

and the porcine arterivirus PRRSV, were selected as controls in this study. The specific peptide-bearing phages could be used as diagnostic reagents in indirect ELISA to differentiate the inactivated SARS-CoV specifically from above-mentioned viruses. As the control phage bearing phage complex from the phage library only had a negligible effect, the current result indicated that the phage-based ELISA was mainly mediated by the foreign peptides displayed on the surface of these phages.

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Competing interest

The authors have no competing interests.

Ethical approval

This experiment has been approved by the Ethical Committee of Northeast Agricultural University.

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