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Original Article



# Sputum Processing Method for Lateral Flow Immunochromatographic Assays to Detect Coronaviruses

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## ABSTRACT

Coronavirus causes an infectious disease in various species and crosses the species barriers leading to the outbreak of zoonotic diseases. Due to the respiratory diseases are mainly caused in humans and viruses are replicated and excreted through the respiratory tract, the nasal fluid and sputum are mainly used for diagnosis. Early diagnosis of coronavirus plays an important role in preventing its spread and is essential for quarantine policies. For rapid decision and prompt triage of infected host, the immunochromatographic assay (ICA) has been widely used for point of care testing. However, when the ICA is applied to an expectorated sputum in which antigens are present, the viscosity of sputum interferes with the migration of the antigens on the test strip. To overcome this limitation, it is necessary to use a mucolytic agent without affecting the antigens. In this study, we combined known mucolytic agents to lower the viscosity of sputum and applied that to alpha and beta coronavirus, porcine epidemic diarrhea virus (PEDV) and Middle East respiratory syndrome coronavirus (MERS-CoV), respectively, spiked in sputum to find optimal pretreatment conditions. The pretreatment method using tris(2-carboxyethyl)phosphine (TCEP) and BSA was suitable for ICA diagnosis of sputum samples spiked with PEDV and MERS-CoV. This sensitive assay for the detection of coronavirus in sputum provides an useful information for the diagnosis of pathogen in low respiratory tract.

**Keywords:** Coronavirus; Diagnosis; Sputum; Chromatography, Sensitivity

## INTRODUCTION

Coronaviruses (CoV) are zoonotic pathogens that infect a variety of species including bat, camels and human (1,2). They are classified into 4 groups: alpha coronavirus, beta coronavirus, gamma coronavirus and delta coronavirus (3). Among beta coronavirus, a novel C lineage virus detected in the Middle East and called Middle East respiratory syndrome coronavirus (MERS-CoV) causing severe lower respiratory tract infection in human, occasionally accompanied by renal disease (3).

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**Conflict of Interest**
The authors declare no potential conflicts of interest.

**Abbreviations**
CoV, coronavirus; DW, distilled water; ICA, immunochromatographic assay; MERS-CoV, Middle East respiratory syndrome coronavirus; NALC, N-acetyl-L-cysteine; ND, not detect; NT, not test; PEDV, porcine epidemic diarrhea virus; PI, protease inhibitor cocktail; RT-LAMP, reverse transcription loop-mediated isothermal amplification; TCEP, tris(2-carboxyethyl)phosphine; TCID50, median tissue culture infectious dose.

**Author Contributions**
Conceptualization: Yeom M, Kim H, Na W, Song D; Data curation: Kang A, Yeom M, Kim H, Yoon SW, Jeong DG, Moon HJ, Lyoo KS; Formal analysis: Yeom M, Yoon SW, Jeong DG, Moon HJ, Lyoo KS, Na W, Song D; Funding acquisition: Song D; Investigation: Kang A, Jeong DG, Lyoo KS; Methodology: Kang A, Kim H, Yoon SW, Jeong DG, Moon HJ, Na W; Resources: Kim H, Yoon SW, Jeong DG; Validation: Moon HJ; Writing - original draft: Kang A, Yeom M, Na W; Writing - review & editing: Na W, Song D.

MERS-CoV was transmitted from animal to human and spread in human population around the world, resulted in 2,494 laboratory-confirmed cases with 858 deaths, since 2012 (4,5). Korea Centers for Disease Control and Prevention reported 186 cases with 38 deaths in Korea, July 2015 (6).

Laboratory diagnosis of MERS-CoV is performed by real-time RT-PCR and reverse transcription loop-mediated isothermal amplification (RT-LAMP) (7-9). These molecular methods of diagnosis provide highly sensitive and specific detection, albeit it is relatively time consuming and labor intensive (9-11).

Antigenic detection of MERS-CoV could be conducted by ELISA and immunochromatographic assay (ICA) (12,13). ICA is used simple and rapid in point of care (11). However, ELISA is labor intensive and need trained personal (14).

ICA for detection of MERS-CoV was developed to be applied to camels for quarantine in field by obtaining nasopharyngeal swabs. The veterinary ICA showed high specificity and sensitivity to nasopharyngeal specimens of confirmed camels cases (13). However, when the ICA was applied to that of human confirmed case, the result presented weakly positive or negative (15). In humans, MERS-CoV mainly replicates and exists in the lower respiratory tract, so sputum samples from the lower respiratory tract are used (16,17). However, the sputum from lower respiratory tract was unlikely to be applied to ICA due to its thick, sticky, pus, frothy, blood-stained, harsh condition.

Recently, many studies have been conducted on the pretreatment of samples to apply these sputum to ICA (18,19). Previous studies have shown that reducing agents were used to dissolve the sputum that has disulfide bond by tris(2-carboxyethyl)phosphine (TCEP) and N-acetyl-L-cysteine (NALC) (20-22).

In this study, optimal conditions were designed with TCEP, NALC, BSA and protease inhibitor cocktail (PI), which are considered to be the most effective among the various pretreatment methods aforementioned. We applied a pretreatment method to the sputum samples spiked with porcine epidemic diarrhea virus (PEDV) of alpha coronavirus and MERS-CoV of beta coronavirus, respectively, and was performed to evaluate whether it is suitable for ICA diagnosis.

## MATERIALS AND METHODS

### Preparation of viruses and sputum samples

The PEDV DR-13 strain (accession No. JQ023161), which belongs in alpha group of corona virus was provided by Green Cross Veterinary Product (Yongin, Korea). The live MERS-CoV, MERS-CoV/KOR/KNIH/002/05(2015) that belongs in beta group of corona virus was provided by Korea Centers for Disease Control and Prevention. Coronavirus-free sputum samples originated from human were provided by Dr. Song in Seoul National University Hospital, and were approved at the South Korea Medical Institutional Review Board (approval number: 1603-016-747). Sputum samples were spiked by volume of 60% with PEDV or live MERS-CoV that have $c_q$ value of 20.15 and 25.26, respectively.

### Reagents

The mucolytic TCEP-HCI (Thermo Fisher Scientific, Waltham, MA, USA) was prepared to different concentration, 10 to 30 mM, using distilled water (DW). Reducing agent, NALC ≥99% (Sigma-Aldrich, St. Louis, MO, USA) was prepared by the minimum concentration for reducing reaction, 40 mM, according to manufacturer specification. A neutralizing agent, BSA fraction V (7.5% solution) (Sigma-Aldrich) was prepared 3.75% and 7.5%. Complete tablets EASYpack PI (Roche, Basel, Switzerland) was prepared to various concentrations using DW.

### Application of mucolytics and neutralizing agent

TCEP, BSA or PI were added to the sputum samples sequentially with volume of 100%, 30%, 6%, of sputum sample, respectively, and incubated for 15minutes at room temperature. Each reagent was replaced with same volume of PBS, accordingly, to compare the efficacy of the each reagent. The processed sputum samples were applied to rapid immunochromatographic kit, PED Ag test kit or MERS Ag test kit (Bionote, Hwaseong, Korea), according to the manufacturer's protocol, and the band intensity was calculated by percentage compared to intensity of control line with MEDISENSOR Gold reader (MEDISENSOR, Daegu, Korea).

### Sensitivity comparison of ICA and real-time RT PCR

PED virus was diluted in 5-, 10-, 50-, 100-, and 1,000-fold. The PED virus was spiked with 60% volume of the sputum samples. The test samples were pretreated sequentially with 10 mM TCEP and 7.5% BSA with 100% and 30% volumes of the sputum samples and incubated for 15 min at room temperature. The non-treat samples were added with PBS in 130% volumes of the sputum samples. As a control samples, PEDV diluted with PBS in the same amount as the test samples were prepared. The sensitivity of ICA by test samples and control samples were compared using real-time RT PCR.

RNA was extracted according to the QIAamp Viral RNA Mini Handbook (Qiagen, Hilden, Germany) for purification of viral RNA from pretreatment samples. Real-time RT PCR was performed using a SensiFAST Probe No-ROX One-Step Kit (Bioline, London, UK) with 20μl final reaction volume containing 4 μl of RNA template, 0.4 μM of each primer (forward 5′-CGCAAAGACTGAACCCACTAATTT-3′; reverse 5′-TTGCCTCTGTTGTTACTTGGAGAT-3′) and 0.2 μM of probe (6-carboxyfluorescein [FAM]-5′-TGTTGCCATTGCCACGACTCCTGC-3′-BHQ1). Thermal cycling conditions included reverse transcription at 45°C for 10 min, initial denaturation at 95°C for 2 min, and 40 cycles at 95°C for 5 s followed by 60°C for 20 s. The data were analyzed using LightCycler 96 system (Roche USA, Nutley, NJ, USA).

## RESULTS

### Intensity enhancement in ICA of alpha coronavirus (PEDV)

In order to break the disulfide bonds in sputum, 10, 20, and 30 mM of TCEP were added to a sputum spiked with PEDV. The intensity of the test line in the immunochromatographic test increased 1.43% on average by treatment TCEP with 10mM, while negative results from treatment TCEP with PBS, 20 and 30mM (**Fig. 1**). To minimize the impact of PEDV detection in the ICA, we concluded that the TCEP should be treated at low concentrations. Two concentrations of BSA were added because the pretreatment conditions should be optimized to acquire the best intensity on the test line. When 3.75% or 7.5% of BSA were added to 10 mM TCEP, the intensity of test line increased 17.45% and 79.89% on average, respectively,

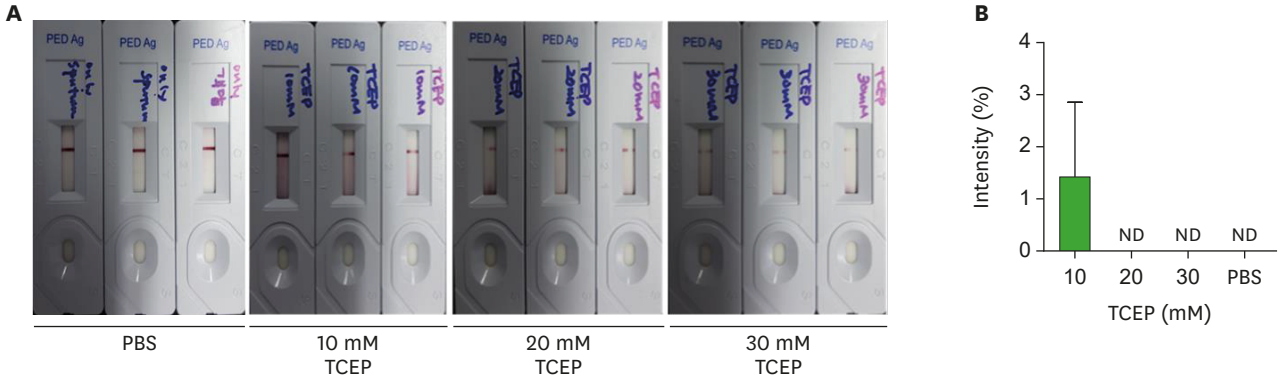


**Figure 1.** Detection of PED virus after treatment PBS or TCEP.
(A) Lateral-flow ICA strip detection of PEDV spiked in sputum sample spiked PEDV and PBS, 10, 20, and 30 mM TCEP, respectively. (B) The intensity of test line was measured with a MEDISENSOR Gold reader. (Average 0%, 1.43%, 0%, and 0%).

while these were similar or decreased in additions of the BSA to 20 or 30 mM TCEP treatment (**Fig. 2**). Under the conditions of 10 mM TCEP and 7.5% BSA, the intensity of test line was compared by concentration of PI. The PI solutions were prepared by concentration of 1×, 2×, 3×, 4×, 5×, and 6×. The intensity of the test line by the PI concentrations (1×, 2×, 3×, 4×, 5×, and 6×) were average of 4.60%, 4.27%, 4.10%, 3.84%, 4.10%, and 3.60%, respectively,

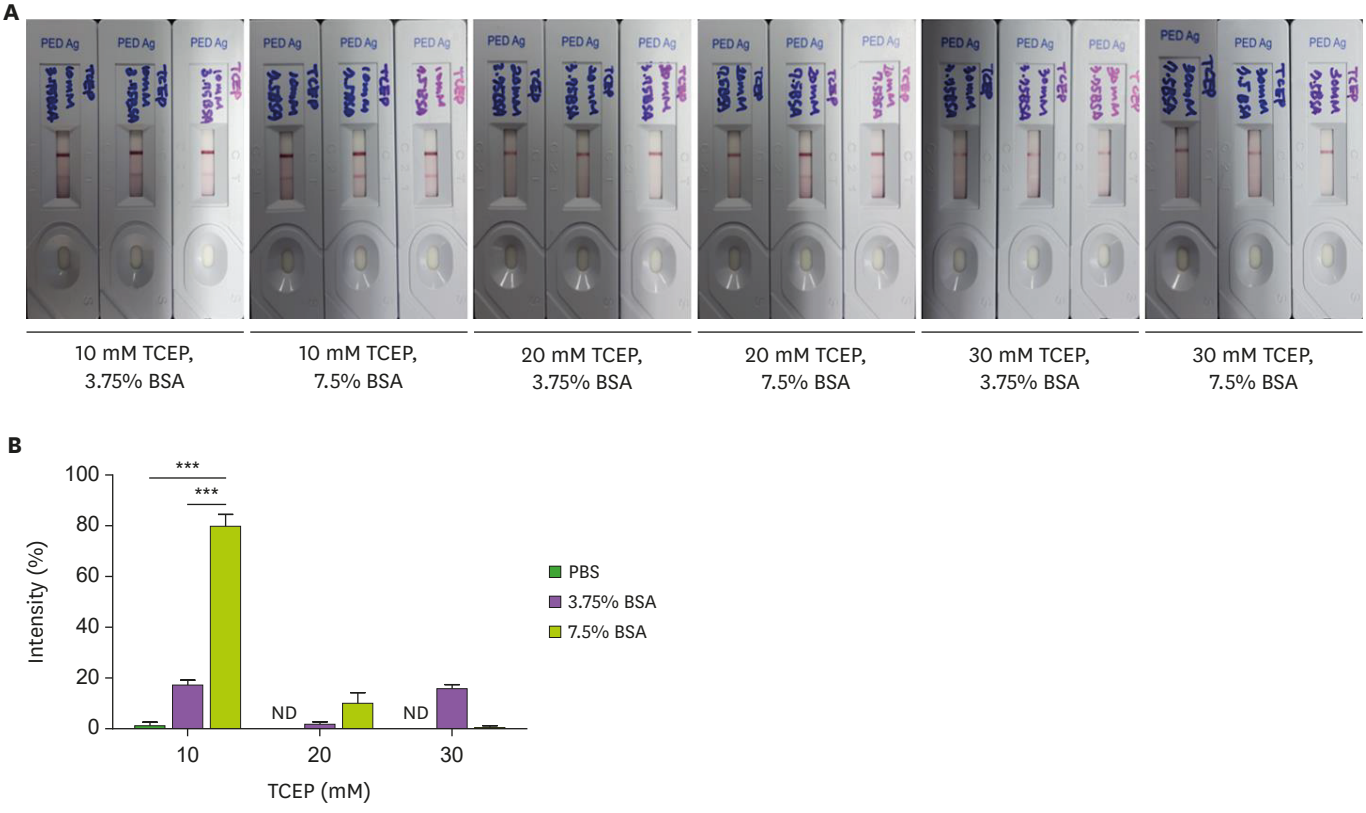


**Figure 2.** Detection of PED virus after treatment TCEP and BSA.
(A) Lateral-flow ICA strip detection of PEDV spiked in sputum sample spiked PEDV with different concentrations of TCEP and BSA, respectively. (B) The intensity of test line was measured with a MEDISENSOR Gold reader (average 17.45%, 79.89%, 2.07%, 10.27%, 16.04%, and 0.71%). Statistical differences were tested in comparison with the 7.5% BSA value at 10mM TCEP.
Significance level by a t-test, ***$p<0.001$.


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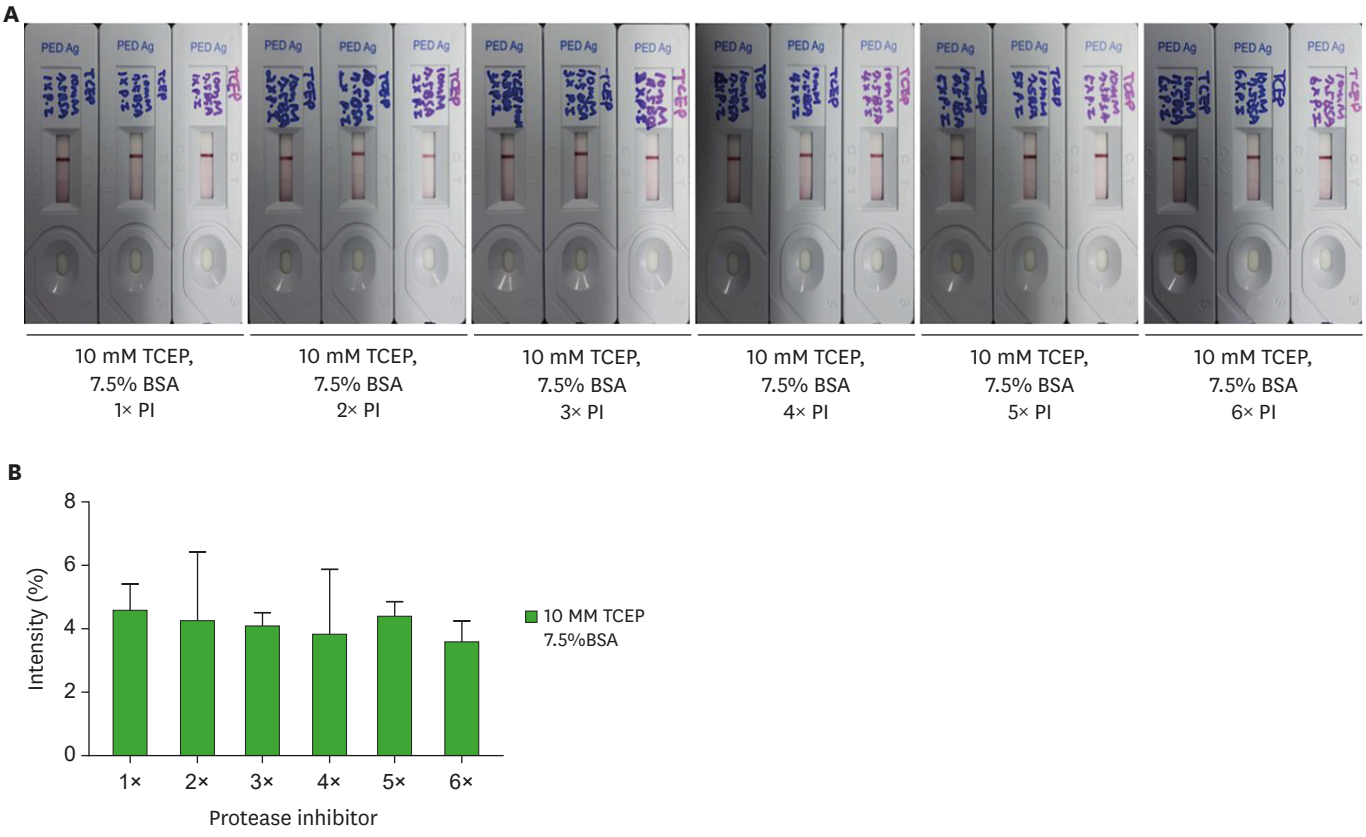


**Figure 3.** Detection of PED virus after treatment TCEP, BSA, and PI.
(A) Lateral-flow ICA strip detection of PEDV spiked in sputum sample spiked PEDV with 10 mM TCEP, 7.5% BSA and different concentrations of PI, respectively. (B) the intensity of test line was measured with a MEDISENSOR Gold reader (average 4.60%, 4.27%, 4.1%, 3.84%, 4.41%, and 3.6%).

which were decreased compared to the 10 mM TCEP and 7.5% BSA. There was no correlation between intensity of test line and the PI concentration to the treatment of 10 mM TCEP and 3.75% (**Fig. 3**).

## Intensity enhancement in ICA of beta coronavirus (MERS-CoV)

To determine optimal pretreatment conditions for virus detection in sputum by ICA, the conditions chosen for PEDV were evaluated in MERS-CoV. Based on the condition of increasing PED diagnosis efficiency in sputum, we tested whether similar results were observed in MERS diagnosis kit. Also, we measured the intensity of ICA test line when sputum sample reacts with NALC, which plays a similar role to TCEP in sputum. The intensity in test line showed an average of 1.77% in 10 mM TCEP alone treatment, and there was no positive signal test line in 40 mM NALC alone (**Fig. 4**). However, when 10 mM TCEP was treated with 3.75% and 7.5% BSA, the intensity of test line increased on average of 4.8% and 63.57%, respectively, compared with samples treated with 10 mM TCEP alone treatment. Also, when 40 mM NALC was treated with 3.75% or 7.5% BSA, the intensity of test line increased on average were 40.75% and 7.42%, respectively. The 10 mM TCEP and 7.5% BSA combination was 24.6% higher than the 40 mM NALC and 3.75% BSA in the intensity of test line (**Fig. 5**). The results presented that the pretreatment combination of 10 mM TCEP and 7.5% BSA improves a PEDV and MERS-CoV detection efficacy.


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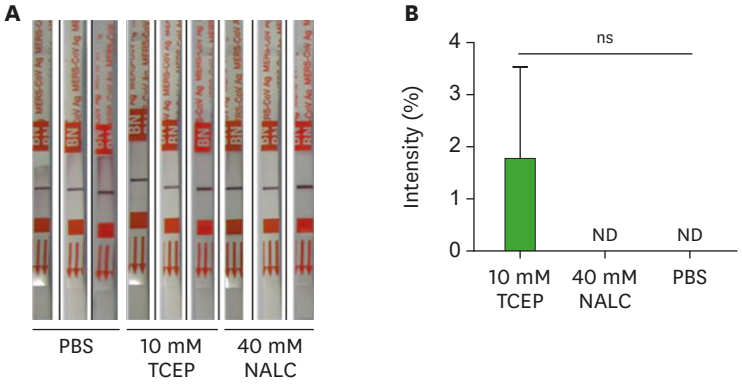


**Figure 4.** Detection of MERS virus after treatment PBS or reducing agents.
(A) Lateral-flow ICA strip detection of MERS-CoV spiked in sputum sample spiked MERS-CoV and PBS, 10 mM TCEP and 40 mM NALC, respectively. (B) The intensity of test line was measured with a MEDISENSOR Gold reader (average 0%, 1.8%, and 0%). Values are means ± SEM.
ns, not significant.

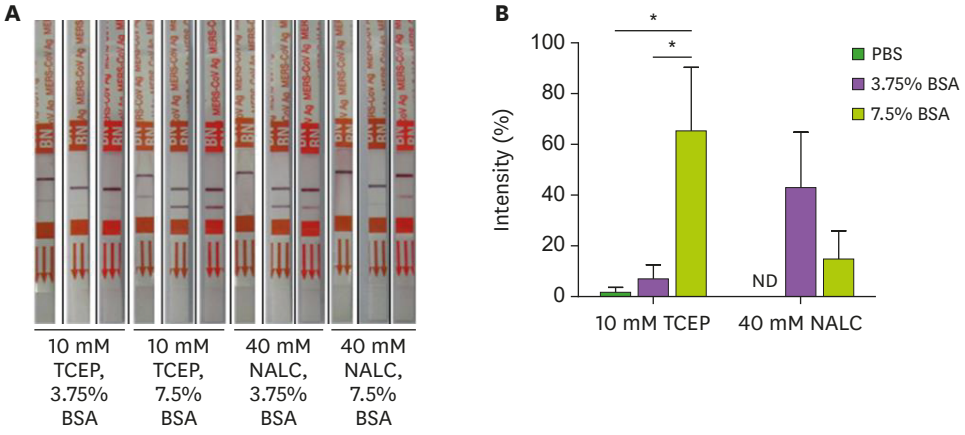


**Figure 5.** Detection of MERS virus after treatment reducing agents and BSA.
(A) Lateral-flow ICA strip detection of MERS-CoV spiked in sputum sample spiked MERS-CoV sequentially with 10 mM TCEP or 40 mM NALC and 3.75% or 7.5% BSA. (B) The intensity of test line was measured with a MEDISENSOR Gold reader (average 1.8%, 6.6%, 65.3%, 0%, 40.7%, and 7.4%). Statistical differences were tested in comparison with the 7.5% BSA value at 10 mM TCEP.
Significance level by a t-test, *$p<0.05$.

## Detection limit comparison

In order to compare the detection limit under optimal condition of pretreatment, diluted PEDV was spiked in the sputum. The PEDV stock was $10^6$ median tissue culture infectious dose ($TCID_{50}$)/ml and was diluted 5, 10, 50, 100 and 1,000 times. The ICA of PEDV for sputum samples with 10 mM TCEP and 7.5% BSA, were compared with real-time PCR, and these results in 3 replicates are summarized in **Fig. 6** and **Table 1**. The non-treat samples, sputum was spiked with diluted PEDV and was added PBS in the same volume as the pretreatment reagents. The test samples, sputum was spiked with diluted PEDV and added with 10 mM TCEP and 7.5% BSA. The control samples were added PBS to the diluted PEDV up to same volume as the test sample. Real-time RT-PCR detection of PED virus in the test, non-treat and control samples were all positive, and $C_T$ values by sputum and pretreat reagents did not show any difference. However, non-treat samples did not flow through the strip in the ICA because had high viscosity, and ICA failed to detect. The detection limit of ICA for the control sample


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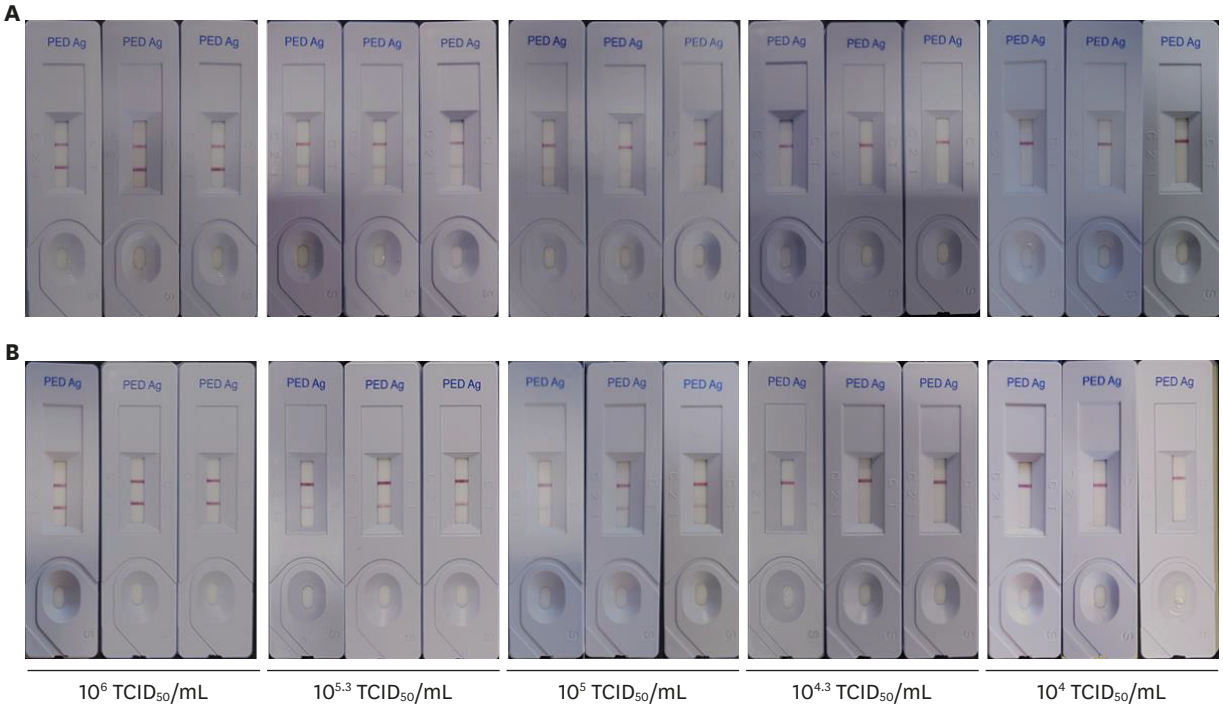


**Figure 6.** Detection of pretreated PED virus spiked sputum samples and diluted PED virus in the same amount.
(A) Test samples; lateral-flow ICA strip detection of PED virus in sputum sample spiked PED virus ($10^6$, $10^{5.3}$, $10^5$, $10^{4.3}$, and $10^4$ $TCID_{50}$/ml) with 10 mM TCEP and 7.5% BSA. (B) Control samples; lateral-flow ICA strip detection of PED virus stock solution in diluted in the same amount as the test samples.

**Table 1.** Comparison of sensitivity ICA and real-time PCR to detect PEDV

| Virus titer ($TCID_{50}$/ml) | Non-treat sample | | Test sample | | Control sample | |
|---|---|---|---|---|---|---|
| | $C_T$* value of real-time RT-PCR | Result of ICA | $C_T$* value of real-time RT-PCR | Result of ICA | $C_T$* value of real-time RT-PCR | Result of ICA |
| $10^{6.0}$ | 15.83 | ND | 15.93 | Positive | 15.09 | Positive |
| $10^{5.3}$ | 17.29 | ND | 18.18 | Positive | 17.91 | Positive |
| $10^{5.0}$ | 18.07 | ND | 19.10 | Positive | 18.83 | Positive |
| $10^{4.3}$ | 21.17 | ND | 22.08 | Negative | 20.59 | Faint band |
| $10^{4.0}$ | 22.01 | ND | 22.27 | Negative | 21.85 | Negative |
| $10^{3.0}$ | 26.88 | ND | 27.83 | NT | 26.01 | NT |

Non-treat sample, sputum sample spiked PED virus with PBS. Test sample, sputum sample spiked PED virus sequentially with 10 mM TCEP and 7.5% BSA.; Control sample, PED virus in diluted in the same amount as the test samples.
*Threshold cycle.

was $10^{4.3}$ $TCID_{50}$/ml. When TCEP and BSA were treated with sputum spiked with PED, ICA showed a detection limit of $10^5$ $TCID_{50}$/ml.

## DISCUSSION

This study investigated the method of pretreatment of the sputum, which could be used to detect the coronavirus by lateral flow ICA in human cases. The structure of the sputum proteins is mainly composed of disulfide bonds, in which folded proteins are formed and responsible for their high viscosity and adhesive strength (23,24). Those features are not suitable for application to ICA based on lateral flow. To modify the properties of a sputum specimens, TCEP and NALC have been used due to their ability to break the disulfide bond

of sputum (8,10,12,13). This study confirmed that the pre-treating of TCEP and NALC can facilitate the application of sputum to ICA, minimizing loss of corona virus.

By using the point-of-care testing through pretreatment of sputum, the time required for diagnosis can be shortened and quarantine can be carried out efficiently. The diagnosis of coronavirus is conducted by real-time RT-PCR, RT-LAMP, reverse transcription recombinase polymerase amplification, ELASA, however, the laboratory diagnoses require professionals who conduct pre-analytic and analytic procedures, and sophisticated equipment (7-12). On the other hand, the ICA can rapidly detect virus antigen and is simple to use with easy to interpret (11). ICA for MERS-CoV in camel's nasal shed presented high sensitivity as previously described (13), while the results through this study revealed that the ICA showed limited sensitivity in nasal shed of MERS-CoV confirmed human cases. It seemed that the viral load in upper respiratory tract is significant lower than that of lower respiratory tract in humans (16,17). It is well known that the receptor for MERS-CoV mainly distributed in lower respiratory tract in human (25,26), which affects the different tropism of MERS-CoV in respiratory tract. For this reasons, the diagnosis for MERS-CoV in human cases, sputum samples from the lower respiratory tract should be tested. However, the sputum is not applicable for the ICA, because the ICA is based on flow strip and requires low viscosity so that the antigen could transport through the strip (27-29).

In this study, the TCEP and NALC were used to break the disulfide bonds of sputum and make it serous enough to flow on ICA. However, the addition of reduction agent alone showed only minor effects and made the need for additional substance. To lessen the reducing strength of TCEP and NALC, blocking agent, BSA, was added, and the values of the ICA test line increased when the reducing agent and BSA were treated together than the reducing agent alone. As the concentration of TCEP increases to the sputum spiked PED virus, the antigen detection rate decreases in the PED detection kit, while the 7.5% BSA was added with 10 mM TCEP, the detection rate of antigen was increased up to 79.89%.

The treatment with TCEP and NALC is not suitable for detecting the antigen of MERS virus, albeit resulted in complete reduction of sputum samples. Additional treatment with BSA was effective in detecting the antigen of MERS virus. The intensity of test line in the sputum spiked MERS virus was the highest at 65.3% when the samples were treated with 10 mM TCEP and 7.5% BSA.

The sputum samples spiked PED virus were pretreated with 10 mM TCEP and 7.5% BSA, and then ICA sensitivity were compared with real-time PCR. The detection limit of ICA was a 10-fold dilution, and that ICA was less sensitive than real-time PCR (more than 1,000-fold dilution). Compared to the ICA of the control samples, the PED detection limit of the pretreated sputum sample ICA was 5-fold lower, but the PED detection results of ICA and real-time RT PCR in the pretreated sputum samples showed a constant trend as the virus was diluted.

In this study, we have performed a diagnostic comparison test using sputum samples spiked corona virus. However, the pretreatment method could be optimized by purity of antibody and its affinity. Also, group of corona virus differed from their structure and biochemistry characteristics. Therefore, the study requires a further study that can provide an useful information of antigen preprocessing prior to lateral flow immunochromatographic assays for antigen detection.

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