ABSTRACT

Aim: to evaluate the specificity of the SARS-CoV N protein-based IgG ELISA assay for detection of immunoglobulin G (IgG) in plasma samples obtained from HIV-1 positive and HIV-1 negative intravenous drug users (IDUs). Methods: the SARS-CoV N gene was cloned into pQE-80L vector, and the constructs were transformed into Escherichia coli BL21. The 6 x His-tagged N protein was expressed by inducing the bacterial cells with isopropyl-1-thio-D-galactopyranoside (IPTG) and purified by Ni-NTA affinity resin. The 6 x His-tagged N protein was used as antigen for ELISA assay and evaluated for the serum samples from patients with SARS positive and the plasma samples from the HIV-1 positive and negative IDUs. Results: all sera samples from patients with SARS positive were the ELISA positive (100% sensitivity). The ELISA assay yielded no positive results of the total 61 HIV-1 negative IDU samples (100% specificity) and two positive results of the total 68 HIV-1 positive IDU samples (97.06% specificity). Conclusion: the specificity of the SARS-CoV N protein-based IgG ELISA assay for the detection of the SARS-CoV N specific IgG in plasma samples from IDUs with HIV-1 positive is, therefore, questionable.

Key words: SARS-CoV, N protein, ELISA, intravenous drug users, HIV-1.
INTRODUCTION

Severe acute respiratory syndrome is a disease caused by a new viral entity called SARS-CoV, initially emerged in Guandong Province, China in November 2002. In February 2003, the virus spread globally including Hong Kong, Singapore, Canada, United States, Ireland. And Vietnam etc. Globally, the virus infected 8096 persons with 774 deaths.

Rapid and early detection of SARS-CoV-infected patients is a critical aspect to prevent the spread of SARS disease. Therefore, the SARS-CoV N protein-based serological assays for detection of antibodies (Abs) were developed, because the N protein is highly immunogenic and earliest expressed, and N protein-anti Abs are most abundantly circulated in high titer during infection. Unfortunately, the assays showed cross-reactivity (false positive) with sera of patients infected with other human coronaviruses besides SARS-CoV, polyclonal antisera of group I coronaviruses, and even, healthy sera. The antigenic similarity of SARS-CoV N protein with N proteins of other human coronaviruses, certain human protein such as IL-11, and proteins of unknown particular pathogens are thought as the causative factors of the cross-reactivity. The evaluation of the cross-reactivity of recombinant SARS-CoV N-based serological assays for different types of specimens has been reported. Here, we reported the potential cross-reactivity of the SARS-CoV N protein-based IgG ELISA assay for plasma samples from the IDUs having a risk behavior to widely possible infections.

METHODS

SARS-CoV Genome RNA and SARS Positive Sera

Preparations of SARS-CoV genome RNA and five SARS positive sera (IgG & IgA positive and IgM negative by IFA [immunofluorescent assay]) were performed at the Robert Koch-Institute in Berlin, Germany under the grant from the European Community DG SANCO and were provided by the European Network for Diagnostics of “Imported” Viral Diseases (ENIVD). The virus, SARS-CoV strain Tor2, was isolated from supernatant of infected Vero E6 cells. The virus and sera were inactivated by heating at 560°C for 1 h and gamma irradiation before transported to our laboratory.

Specimens

Twenty healthy sera were used to determine the cutoff value of the ELISA assay. The cut-off OD450 was defined as mean and five standard deviations (X+5S.D.). One hundred and twenty-nine plasma samples (61 HIV-1 negative and 68 HIV-1 positive by HIV-1 ELISA) from IDUs in Jakarta, Indonesia were collectively obtained from Ministry of Health, Directorate General Communicable Disease Control of Indonesia. All IDUs were not more than 21 years old. The HIV-1 positive IDUs were not treated by antiretroviral drugs (ARDs) until blood taken and were not categorized at AIDS stage. All sera or plasma samples were stored at -80°C until used and heated at 560°C for 30 min before used.

Cloning, Expression, and Purification of Recombinant SARS-CoV N Protein

RT-PCR was performed with forward (5' GCG GAT CCA TGT CTG ATA ATG GAC CCC AA-3') and reverse (5'-ACG TCG ACT TAT GCC TGA GTT GAA TC-3') primers. N gene was cloned into BamHI and SalI sites of prokaryotic expression vector pGEX-6P1 (Amersham Pharmacia) and subcloned into pQE-80L vector (Qiagen). Sequence of insert and in frame was confirmed by DNA sequencing. The pQE constructs were transformed into Escherichia coli strain BL21. The 6x His-tagged N protein (the recombinant protein) was expressed by inducing bacterial cells with 0.2 mM isopropyl-1-thio-D-galactopyranoside (IPTG) at room temperature for 4 h. Bacterial pellets were sonicated and solubilized in lysis buffer (1.5% N-lauroylsarcosine, 1% Triton X-100, 150mM NaCl, 10 mM imidazol, and 10mM Tris, pH 8.0). Finally, purification of the 6 x His-tagged N protein was performed using Ni-NTA chromatographic method (Qiagen) according to the manufacturer’s instructions.

IgG ELISA

Wells of microtiter plates (Corning Costar) were coated with 100 µl of 0.1 M carbonate buffer pH 9.6 containing 100 ng of purified recombinant protein at 1-2°C for 16-18 h. Thereafter, the wells were washed fifth with phosphate-buffered saline pH 7.4 containing 0.05% Tween 20 (PBS-T) followed by incubation with PBS-T containing 1% BSA at 37°C for 1 h. After removing the blocking solution, 50 µl of 1:100 plasma or
serum diluted in PBS-T containing 0.05% gelatin (PBS-TG) was added to the wells and incubated for 30 min at 37°C. The wells were washed again and incubated for 30 min at 37°C with 100 µl of anti-human IgG biotin-labeled goat antibody (Chemicon) diluted at 1:4000 in PBS-TG. The wells were washed again and incubated for 30 min at 37°C with 12.5 ng of peroxidase-conjugated streptavidin diluted in PBS-TG. After five further washes, 200 µl of substrate solution (0.5 mg/mL 0-Phenylenediamine Dihydrochloride in 0.1 M Citrate phosphate buffer) was added to each well and incubated for 15 min at room temperature in dark. The absorbance was examined at 450 nm using Microplate Reader 550 (Bio-Rad).

Western Blot

The Western blot was used for confirmation of the ELISA positive results. One hundred ng of the purified N protein was run on sodium dodecyl sulfate-polyacrylamide gel and transferred on to a nitrocellulose membrane. After blocking with 1x tris-buffered saline (TBS) containing 0.05% Tween 20 [Bio-Rad] and 0.5% gelatin [Bio-Rad] at 4°C for 16-18 h, the membranes were incubated with a 1:100 dilution of plasma samples at 25°C for 2 h. The membrane was washed twice with 1x TBS containing 0.05% Tween (TBS-T) for 5 min. The membrane was then reacted with a 1:5000 dilution of biotinylated rabbit anti-human IgG antibody at 25°C for 1 h. The membrane was washed again twice with TBS-T for 5 min and reacted with 0.25 µg of horseradish peroxidase-conjugated streptavidin diluted in 1x TBS containing 0.02% Tween and 0.1% gelatin at 25°C for 1 h. After two washes with TBS-T for 5 min, the immunocomplexes were detected with DAB Solution (per 100 mL 1x TBS: 60 mg 3,3’-diaminobenzidin [Sigma]; 100 µL 30% H2O2) and incubated at 25°C for 2-5 min. The reaction was terminated by adding sterile distilled water.

Homology Analysis

The amino acids of the SARS-CoV N protein in this study were compared with those of N proteins of other human coronaviruses that were retrieved from GeneBank with following accession numbers: human coronavirus 229E [HCoV-229E] (NC_002645, X51325, AF304460, and HOBNC229E), HCoV-HKU1 (NC_006577, DQ778921, DQ415914, and DQ415913), HCoV-NL43 (NC_005831, FJ211861, EF081296, and DQ445912), and HCoV-OC43 (NC_005147, AY903460, AY903459, and AY391777).

RESULTS

To develop an ELISA for the detection of the SARS-CoV N protein specific antibody, the full length of SARS-CoV N protein (422 amino acids) with an amino terminal 6 x Histidin-tag was expressed in E. coli BL21 and purified to homogeneity.

The mean OD450 for 20 healthy sera was 0.507 with standard deviation of 0.40; the cutoff OD450 was 0.709. Using this cutoff, the ELISA assay showed the 100% sensitivity (5 of 5 SARS positive sera) (Figure 2). For specificity, the ELISA assay was evaluated using plasma samples from the 68 HIV-1 positive and the 61 HIV-1 negative IDUs. Of the 68 HIV-1 positive IDU samples, the assay showed two positive results (97.06%), while all 61 HIV-1 negative IDU samples showed no positive result (100%) (Figure 2). The two ELISA positive and 5 other ELISA negative samples from HIV-1 positive IDUs were confirmed by Western blot assay. The results of Western blot assays showed the same results as the ELISA assay (Figure 3). Therefore, the overall specificity of the ELISA assay for all IDU samples was 98.45% (2 of 129).

Amino acid sequences of SARS-CoV N protein in this study was compared with those
of N proteins of other human coronaviruses to prove the possibility of antigenic similarities that might cause the cross-reactivity of the SARS-CoV N protein with antibodies specific other human coronavirus N proteins (Figure 4). The homology analysis showed that there were homology regions at N-terminal (Figure 4) but none of the homology at C-terminal domains (data not shown).

**DISCUSSION**

Until 16 June 2003, 7 suspects and 2 probable cases of SARS were reported from Indonesia. The suspected cases had been confirmed and did not fulfill the SARS definition. The two probable patients of SARS had just returned from Hongkong and Singapore. The patients were isolated properly and none of any SARS-CoV transmission from both patients was reported. Since then, there have been no suspect cases reported in Indonesia. Therefore, in this study we supposed that plasma or serum samples obtained from Indonesia could not be in contact with SARS-infected patients and should be SARS-CoV negative.

As shown in Figure 2, the ELISA assay showed two positive results for the HIV-1 positive IDU samples and none of positive results for the HIV-1 negative IDU samples. The positive results had been confirmed by Western blot showing also positive test results (Figure 3). The results might be false positive caused by various factors. We first highlighted the HIV-1 infection as a causative agent causing the positive false indirectly; it means that the people with positive HIV-1 have abnormal immunity responses able to induce autoantibody responses. Another article it was reported that the SARS-CoV N protein-anti antibodies can cross-react with IL-11, indicating possible cross-reactivity between the SARS-CoV N protein and sera obtained from persons having certain autoantibody to IL-11.

Another possible explanation of the cross-reactivity is the antigenic similarity of the SARS-CoV N protein with other human coronavirus N proteins. The phylogenetic analyses and sequence comparisons show that SARS-CoV has an approximate 25–30% identity with other members of the coronavirus family. The homology at N-terminal domain might cause
the false positive tests resulted from patients that are previously exposed towards other human coronaviruses. Previous reports showed that the N-terminal domain of SARS-CoV N protein (N195) did not cross-react with antibodies against IBV, TGV, and canine coronavirus. Another study showed that the use of C-terminal SARS-CoV N protein for antibody detection in clinical sample and in healthy sera could improve the specificity of assay compared with the use of full-length SAR-CoV N protein. To prove the evidences, analysis of amino acid homology was performed and showed homology regions at N-terminal domain (Figure 4) and no homology in C-terminal region of the N proteins (data not shown); indicating that the N-terminal domain of SARS-CoV N protein is potential to cross-react with antibody against the N proteins of HCoV-229E, HCoV-HKU1, HCoV-NL63, and HCoV-OC43. In this regard, Woo and colleagues showed the cross-reactivity of SARS-CoV N protein with clinical serum samples from patients infected by HCoV-229E and HCoV-OC43. In addition, the cross-reactivity of the antibodies to the 6 x His tag of the recombinant N protein was also analyzed by Western blot assays using the recombinant N protein or another recombinant protein shared their amino terminal 6 x His tag. The study indicated that the Western blot positive results were not caused by affinity of antibodies with the 6 x His tag (data not shown). Also, in another study it was reported that an amino terminal 6 x His tag did not influence the sensitivity or specificity of the serological detection of epizootic hemorrhagic.

To our knowledge, there is no information regarding the specificity of the SARS immunodiagnostic assays on infected HIV-1 people not suffering immunodeficiency from AIDS yet. For patients with AIDS, Chen and colleagues reported that 19 AIDS patients, who were hospitalized together with 95 SARS patients on the same floor, were negative for antibodies to SARS virus. However, it should be noted that patients with AIDS could have abnormal humoral and cellular immune responses that might influence the result of the serological assays. In this study, we tested IDUs with HIV-1 positive and HIV-1 negative but not people with AIDS stage. The ELISA OD450 of HIV-1 negative samples were more compact than that of HIV-1 positive samples (Figure 2). The latter may reflect an altered immune response or different disease stage possibly caused by HIV-1 infection.

CONCLUSION

We successfully developed the IgG ELISA assay for diagnosis of SARS disease and tested the assay for the samples from SARS-CoV infected cases and from the HIV-1 positive and HIV-1 negative IDUs. Larger number of SARS-CoV infected cases are required to confirm our finding. The HIV-1 negative IDUs showed no ELISA positive results, while the HIV-1 positive IDUs showed the two ELISA positive results. The specificity of the assay for HIV-1 positive people is, therefore, questionable.

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REFERENCES


