Development of an Enzyme-Linked Immunosorbent Assay-Based Test with a Cocktail of Nucleocapsid and Spike Proteins for Detection of Severe Acute Respiratory Syndrome-Associated Coronavirus-Specific Antibody

Luis G. Giménez, Jose Rojas, Almudena Rojas, Joaquín Mendoza, and Ana G. Camacho*

Laboratorios Vircell, SL, Granada, Spain

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A new enzyme-linked immunosorbent assay (ELISA)-based immunoglobulin G (IgG)-plus-IgM antibody detection test for severe acute respiratory syndrome (SARS) has been developed by using a cocktail of four recombinant polypeptides as the antigen. These recombinant fragments were designed as parts of two different structural proteins from SARS-associated coronavirus (SARS-CoV). One recombinant polypeptide, S251-683, was designed as part of the spike glycoprotein, and the other three polypeptides comprised almost the whole nucleocapsid protein, avoiding the last 25 C-terminal amino acids. Immunization with a cocktail of these four polypeptides yielded a specific polyclonal antibody that is able to recognize SARS-CoV-infected cells by an immunofluorescence assay. This polypeptide cocktail was also used to set up an ELISA-based IgG-plus-IgM antibody detection test, which showed 99% specificity and 90% sensitivity upon evaluation using sera from 100 healthy negative controls and 20 SARS patients. Separate immunoreactivity assays with each recombinant polypeptide demonstrated that a combination of N and S protein fragments was more suitable than the individual peptides for developing a serological assay for SARS-CoV.

A new coronavirus (CoV) (order Nidovirales, family Coronaviridae, genus Coronavirus) has been implicated as the causal agent of severe acute respiratory syndrome (SARS) (3, 20). Its single-stranded plus-sense RNA genome of ~30 kb contains 23 putative open reading frames, including four major structural proteins that are common to all known CoVs: the spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins (12). The control of an epidemic requires a rapid and accurate diagnostic technique that can promptly identify the infection and allow its treatment and the implementation of infection control measures, including the isolation of cases and the management of contacts to prevent further transmissions. The development of a sensitive and highly specific diagnostic kit that is effective at early stages of SARS is of great interest because of (i) the highly contagious and acute nature of the disease, (ii) the stress caused to individuals by unnecessary quarantines during an epidemic, and (iii) the absence of effective anti-SARS therapy. However, the level of virus excretion is comparatively low during the initial phase of SARS (15); therefore, early diagnosis requires a highly sensitive test that can detect the low levels of viral genome or viral proteins present during the first few days after SARS onset. Several molecular assays have been designed to provide an early diagnosis, based on the detection of specific RNA sequences by PCR. However, RNA extraction protocols are not straightforward and may produce RNA preparations that are not useful for reverse transcription if the protocols are not properly followed, yielding false-positi-
TABLE 1. Oligonucleotide primers used for RT-PCR amplification of recombinant fragments of nucleocapsid and spike proteins from SARS-CoV

| Primer  | Sequence | Position (bp) |
|--------|----------|---------------|
| N1-5'  | ATGTCTGATAATGGACCAATC | 1–23 |
| N1-3'  | TCCCTAGTTGCAACCTATAC | 394–397 |
| N2-5'  | AAGAAGGCATCTGTAGGGTGC | 382–404 |
| N2-3'  | GCAGCATAAGCCGGACGGACT | 652–673 |
| N3-5'  | ATGGCATTGGAATTCGTTCTC | 646–667 |
| N3-3'  | ATGTCACCGGGCAAGAAGAG | 1181–1202 |
| S251-683-5' | TCAGGTGCACTTTATGGTGCG | 753–785 |
| S251-683-3' | TCTAGGGGCTAAGCTTGCCAT | 1334–1366 |
| TCATACATC |          |          |

* Specific oligonucleotide primers were designed from the sequence of the SARS-CoV genome in the GenBank nucleotide sequence database (accession number AT287841) (20).

Polyclonal antibody against SARS-CoV. The polyclonal antibody was prepared by immunizing a rabbit with an intramuscular injection of 200 μg of protein (50 μg of each recombinant polypeptide: N1, N2, N3, and S251-683) in 1 ml of an emulsion containing 50% Freund’s complete adjuvant. The same dose was repeated after 15 days, and the same dose prepared in Freund’s incomplete adjuvant was injected at 15 days after the second dose. The serum used corre-
sponded to a blood sample drawn 3 months after the third injection.

IFA. The immunofluorescence assay (IFA) used SARS-CoV-infected Vero-E6 cells from a commercial SARS-CoV IFA kit (Euroimmun). Briefly, the rabbit polyclonal serum obtained was first purified by protein A affinity chromatography (HiTrap protein A HP, Amersham BioLabs) and then labeled with fluorescein isothiocyanate (Sigma). A 1:1,000 dilution of the fluorescein-labeled rabbit poly-
clonal antibody was incubated for 30 min with SARS-CoV-infected cells. Non-
infected cells were used as a control. After a wash, the reaction was visualized by fluorescence microscopy.

ELISA measurement. Microtiter plates were coated with a mixture of the four recombinant polypeptides, diluted in phosphate-buffered saline (PBS) at a concen-
tration of 1 to 5 μg/ml each (3 μg/ml N1, 2 μg/ml N2, 1 μg/ml N3, and 5 μg/ml S251-683), and were incubated overnight at room temperature. Plates were blocked with newborn calf serum, washed, and then incubated with sera from SARS patients or healthy controls (both at 1:100) in PBS containing newborn calf serum for 45 min at 37°C. After a wash, a 1:100,000 dilution of peroxidase-conjugated goat anti-human immunoglobulin G (IgG) plus IgM (Jackson) was added and incubated at 37°C for 30 min. Finally, the peroxidase reaction was visualized by using a tetramethylbenzidine-hydrogen peroxide solution as a sub-
strate (Neogen Corporation). ELISAs with the single recombinant proteins, each applied on a 96-well plate, were also performed on six SARS serum samples. The same antigen dilutions were used, and the protocol described above was followed.

RESULTS

RT-PCR amplification and expression and purification of the recombinant proteins. The cDNA synthesized by RT-PCR was cloned into the expression vectors. Four different polypep-
tides were prepared; three corresponded to the nucleocapsid protein of the virus, and the fourth corresponded to the spike protein. The amplified fragments were first cloned into pR-
SET, but several problems related to overexpression of the heterologous genes appeared. Therefore, three of the frag-
ments (N1, N2, and S251-683) were cloned into pET-15b, a T7 system expression vector, which increased production by as much as 10 to 15% (22). Lysates of the induced cells displayed the heterologous proteins, which were not present in non-
duced cells (data not shown). Densitometric scanning of stained gels showed that the levels of induced proteins were approximately 10, 5, 15, and 12%, respectively, of the levels of soluble protein in E. coli containing the S251-683, N1, N2, and N3 expression constructs.

A high protein yield was obtained by treating the bacterial pellets with a buffer containing 8 M urea, followed by strong probe sonication. Briefly, a clarified extract of E. coli Rosetta
BL21(DE3) overproducing N1, N2, N3, or S251-683 was adsorbed in batch mode to an affinity resin, purified by using a 7-ml column (diameter, 0.7 cm; height, 5 cm) equilibrated with buffer A (50 mM Tris-HCl [pH 7.5], 100 mM sodium phosphate, 8 M urea, 0.1% Emulphogen, 10 mM imidazole, and 1 M NaCl) at pH 8, and washed with 10 column volumes of buffer B (50 mM Tris-HCl [pH 7.5], 100 mM sodium phosphate [pH 7.0], 8 M urea, 0.1% Emulphogen, 20 mM imidazole, and 1 M NaCl) at pH 8. Recombinant proteins were eluted with 5 column volumes of buffer C (50 mM Tris-HCl [pH 7.5], 100 mM sodium phosphate [pH 7.0], 8 M urea, 0.1% Emulphogen, 250 mM imidazole, and 1 M NaCl) at a flow rate of 6 ml/h. This procedure yielded highly purified proteins. A single band was obtained for each protein, as shown in Fig. 1.

N1, N2, N3, and S251-683 peptides showed similar antigenicities in an immunized rabbit. Western blot analysis of the polyclonal serum from the rabbit immunized against the mixture of the four recombinant polypeptides showed that all recombinant fragments reacted with the antibody in a very similar manner (Fig. 2). The polyclonal antibody was also subjected to a separate ELISA for each recombinant polypeptide, and the results obtained were consistent with those of Western blotting, with S251-683 showing a slightly greater antigenic capacity than the N protein fragments (in order of highest to lowest antigenicity, N2, N3, and N1) (data not shown). Several other pathogen antigens were also tested with the rabbit polyclonal serum (Chlamydia pneumoniae, Mycoplasma pneumoniae, Coxiella burnetii, cytomegalovirus, adenovirus, influenza A virus, influenza B virus, parainfluenza virus, antinuclear antibodies, or rheumatoid factor). These sera were also validated by IFA as negative-control samples for SARS-CoV. No cross-reactivity was detected, since none of the serum samples were positive (Table 2).

The polyclonal antibody induced by the polypeptide mixture showed high reactivity to SARS-CoV-infected cells. The rabbit polyclonal serum was incubated with SARS-CoV-infected and noninfected cells and visualized by immunofluorescence. Figure 3 shows that cells infected with the virus had intact nuclear membranes and fluorescence signals located in the cytoplasm. No immunofluorescence was observed in noninfected cells.

Recombinant-protein-based ELISA for antibody detection. A mixture of the four recombinant polypeptides was used as a coating antigen to develop an ELISA-based IgG-plus-IgM test for the detection of S- and N-specific antibodies against SARS-CoV. The assay specificity was studied by using serum samples from 86 healthy IFA-validated controls (dilution, 1:100), and only 1 was above the selected cutoff value, giving a specificity of 99%. Diluted (1:100) sera from 20 SARS patients (clinically diagnosed and IFA validated) were studied by using the S- and N-based ELISA, and 2 samples were below the cutoff value, giving a sensitivity of 90% (Fig. 4). The specificity of the assay was also tested using sera from 14 individuals infected with non-SARS-related respiratory viruses (adenovirus, respiratory syncytial virus, influenza A virus, influenza B virus, and paramyxovirus), antinuclear antibodies, or rheumatoid factor. These sera were also validated by IFA as negative-control samples for SARS-CoV. No cross-reactivity was detected, since none of the serum samples were positive (Table 2).

All control serum samples (n = 100) (data not shown) but only 6 SARS patient serum samples could be tested using the
recombinant polypeptides separately (Table 2). Regarding their individual sensitivities, S251-683 was more immunogenic than the N polypeptides, and N2 was more immunogenic than N3 and N1, which were equal to each other. With respect to their specificities, recombinant fragment N1 was responsible for the false-positive result obtained.

**DISCUSSION**

Four recombinant polypeptides corresponding to two different structural proteins were evaluated for use in a SARS-CoV diagnostic kit.

In this study, a fragment of spike glycoprotein between amino acids 251 and 683 was selected by means of bioinformatic analysis (19). The S fragment overlaps with several regions reported to contain linear epitopes. Thus, Lu et al. (14) identified the C domain (bp 1323 to 2100) ($S_{438-680}$) as the major immunodominant domain in S protein, and Zhou et al. (27) found that S-protein residues 485 to 625 elicited neutralizing antibodies against the virus. A cocktail of these recombinant antigens was used to produce a polyclonal antibody in order to test their capacities to trigger an immune reaction that recognized the native antigen. It was demonstrated that this antibody was able to detect SARS-CoV by immunofluorescence of infected cells, showing that the functional antigenicities of the recombinant polypeptides were similar to those of their native counterparts.

These recombinant polypeptides were then used to develop an ELISA-based IgG-plus-IgM antibody detection test. Only 20 sera from clinically diagnosed SARS patients were available for these studies. Assays performed with the combination of all four recombinant polypeptides, using sera from healthy donors with no exposure to SARS-CoV as controls, showed a sensitivity of 90% and a specificity of 99%. One possible explanation for the two false-negative results may be that some essential linear antigenic sites are located outside the selected spike protein fragment, since polypeptide S251-683 covers less than one-third of the S protein sequence. Furthermore, the immunogenicity of the recombinant S protein in SARS-CoV may be lower than that of its native counterpart due to effects of the expression system used to overproduce the recombinant polypeptide, with the possible loss of essential conformation-and/or glycosylation-dependent epitopes. The N protein is reported to be free of glycosylation sites and does not appear to change its immunological characteristics, even when expressed in a prokaryotic system (17). Nevertheless, the heavy glycosylation of the spike protein with mannose and/or hybrid oligosaccharides (7) may make it a suitable candidate for use in a eukaryotic rather than a bacterial system.

However, the separate analysis of each polypeptide with six SARS-CoV-positive serum samples showed that the S251-683 fragment (reactive with three of six sera) was less immunogenic than the nucleocapsid protein (reactive with five of six sera due to the additive results of the three fragments). However, the spike protein fragment reacted with one serum sample (sample 3) that did not react with the N antigen. Similar findings have been published by Woo et al. (24) and Yu et al. (25), who concluded that a recombinant N-based IgG ELISA was more sensitive than a recombinant S-based IgG ELISA for the serodiagnosis of SARS-CoV. ELISAs with polypeptides obtained from the nucleocapsid protein showed that N2 was the most immunogenic region. It should be taken into account that these studies used only six positive serum samples; therefore, these results should be confirmed with a more representative number of SARS-CoV-positive sera.

**TABLE 2. Analysis of the sensitivity and specificity of a recombinant S- and/or N-based ELISA for detection of IgG and IgM antibodies in samples of SARS-CoV-positive and -negative sera**

| Fragment | Result with the following SARS patient serum sample$^a$ | No. of SARS-CoV-negative serum samples testing positive$^b$ | Sensitivity (%) | Specificity (%) |
|----------|------------------------------------------------------|--------------------------------------------------------|----------------|----------------|
|          | Blood donor sera (n = 86) | Cross-reaction sera (n = 14) |                  |                |
| N1       | – – – + – + | 1 0 | 33.3 | 99 |
| N2       | + + – – – – | 0 0 | 50  | 100 |
| N3       | + + + – – – | 0 0 | 33.3 | 100 |
| S2$^d$   | + + + + + + | + 0 | 50  | 100 |
| Mixture  | + + + + + + | + 0 | 100 | 99 |

$^a$ + and –, results above and below the cutoff, respectively.

$^b$ Negative controls comprised 86 serum samples from healthy blood donors, of which 1 gave a false-positive result, and 14 SARS-CoV-negative sera from individuals infected with non-SARS-related respiratory viruses, antinuclear antibodies, or rheumatoid factor, which were used for cross-reaction experiments.

$^c$ Data correspond to analyses of the individual recombinant polypeptides and the combination of all of them for only six SARS-CoV-positive serum samples.

$^d$ Fragment S251-683 of spike protein.
No direct serology was performed against other human CoV strains in the cross-reaction assays, but a large percentage of the serum samples from our negative controls probably contain antibodies against these highly prevalent viruses (16). Thus, human CoVs are reported to be responsible for around 30% of all common colds (9). In fact, this may explain the false-positive result we obtained. Since cross-reactivity with the N1 polypeptide has been identified, it may be attributable to a highly conserved N111–118 motif described by Vlasova et al. and present in the N proteins of all CoVs (23). Although only one false-positive serum specimen was obtained from unexposed individuals (1%), the potential for cross-reactivity between SARS-CoV and other human CoVs was not directly assessed in this study and remains a concern.

Analysis with our ELISA showed that the recombinant polypeptides had different levels of immunogenicity with the SARS-CoV-positive sera, and one specific mixture of all four recombinant proteins demonstrated higher sensitivity and specificity in detecting antibodies in sera from patients. Hence, we can report a new ELISA-based IgG-plus-IgM antibody detection test for the diagnosis of SARS. Due to the high degrees of pathogenicity and infectivity of SARS-CoV for humans, detection of IgM and IgG antibodies may be the method of choice for this serodiagnosis. We have increased the sensitivity and specificity of the assay by using a cocktail of SARS-CoV N and S proteins. Furthermore, the use of protein fragments or polypeptides instead of whole recombinant proteins for antibody detection may in part resolve the issue of potential cross-reactivity with proteins of other human CoVs.

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