Recombinant Protein-Based ELISA and Immuno-Cytochemical Assay for the Diagnosis of SARS

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A new Coronavirus (SARS-CoV) is the aetiological agent of the severe acute respiratory syndrome (SARS). Because of the critical role played by serological assays for SARS diagnosis, an in-house ELISA based on SARS-CoV recombinant antigens was developed. The SARS-CoV nucleocapsid protein (N), three N fragments (N1, N2, and N3) and the intraviral domain of the membrane protein (M2) were cloned and expressed in Escherichia coli as histidine-tagged proteins. Six reference sera from SARS patients were used to detect virus-specific IgG in an ELISA using each recombinant protein as coating antigen. High-titre positive reactions were detected in all SARS positive sera. The specificity of the assay appears to be high as no positive reaction was detected in the sera of 20 healthy subjects and 73 patients with non-SARS, low-tract respiratory infections.

Specific hyper-immune sera to SARS-CoV and the recombinant proteins, N, N1, N2, N3, and M2 were also generated in mice and rabbits. The specificity of these sera was confirmed by an immunocytochemical assay on biochips of SARS-CoV infected and uninfected cells. J. Med. Virol. 76:137–142, 2005. © 2005 Wiley-Liss, Inc.

KEY WORDS: SARS; Coronavirus; diagnosis; antibodies; serology

INTRODUCTION

An outbreak of severe acute respiratory syndrome (SARS) swept the world in early 2003, spreading from China to several countries in different continents, and causing more than 8,000 cases with roughly 14%—18% mortality rate [Donnelly et al., 2004]. A new Coronavirus (SARS-CoV) is the etiological agent of this syndrome, accordingly, molecular, virological, and serological tests have been developed for its identification [Chan et al., 2004; Poon et al., 2004b]. Although retrospective in nature, serological tests are of utmost importance either to confirm or rule out infection by the virus and for seroprevalence studies. In this context, the viral nucleocapsid protein N and the membrane protein M are recognized as useful antigens to monitor antibody response in other coronavirus infections [Ndifuna et al., 1998; Che et al., 2003; Shi et al., 2003; Leung et al., 2004; Liu et al., 2004; Woo et al., 2004].

Recombinant proteins are potentially inexpensive, safe, and easy to standardize serological reagents. They also represent the safest way to prepare the viral antigens since they can be obtained without propagation of live virus, a fact of considerable importance in view of the recognized capacity of SARS-CoV to infect laboratory workers. Thus, for SARS diagnosis and seroprevalence studies, an in-house ELISA was developed in our laboratory acting as National Reference Center for Infectious Emergences. The assay is based on the use of SARS-CoV recombinant N and M structural proteins as coating antigens. Purified N and M proteins were also used to generate specific polyclonal antibodies for detection of SARS-CoV and its antigens in clinical specimens.

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MATERIALS AND METHODS

Recombinant DNA Manipulation

The N and M full-length genes were generated by RT-PCR technology from a 10 μl sample containing heat and gamma-irradiated cell culture supernatant of Vero E6 cells infected with the SARS-CoV isolate Frankfurt 1 [Thiel et al., 2003]. The oligonucleotides used are listed in Table I. The BamHI and PstI sequences were added at the 5′-ends of each forward (FW) and reverse (RV) primers, respectively. The BamHI-PstI restricted cDNAs were cloned in pQE-30 (QIAGEN) and introduced by transformation in the JM109 Escherichia coli cells, obtaining the constructs p-N and p-M, respectively. The proteins were expressed as N-terminal MRGS(H)6 tag proteins. The NH2-terminus (1–117 amino acids, aa), the central-domain (110–340 aa), and the COOH-terminus (333–422 aa) of the N protein, and the cytoplasmic-domain (138–222 aa) of the M protein, were also cloned in pQE-30 obtaining the constructs p-N1, p-N2, p-N3, and p-M2, respectively. Numbers refer to the N (AAP33707) and M (AAP33701) EMBL sequences.

Protein Purification

The recombinant proteins were purified by affinity chromatography on Ni-NTA resin using a denaturing protocol according to the manufacture instructions (The QIAexpressionist). All proteins were solubilized by 8 M urea in PBS, except the protein N, which was solubilized by 6 M guanidine hydrochloride in PBS. Protein purity and quantity were analyzed by SDS–PAGE followed by staining with Coomassie blue and by standard method (Lowry), respectively. Protein identity was confirmed by Western blotting using the anti poly-histidine antibody (Sigma-Aldrich, St. Louis, MO).

Human Sera

The six SARS-CoV-positive sera were collected at the Robert Koch Institute in Berlin, Italy acting within the European Network for Diagnostics of Imported Viral Diseases (ENVID). They were kindly obtained from donors and 73 patients affected by viral (non-SARS) or bacterial acute respiratory syndromes served as controls.

ELISA

The SARS-CoV recombinant proteins (0.125 μg/well), in denatured form, were adsorbed in carbonate buffer (pH 9.4) into 96-well microtiter plates overnight at 4°C, then incubated with twofold dilutions of each serum, starting from a 1:600 and 1:2,000 dilutions for N1, N2, N3, and M2-based and N-based ELISA, respectively. Incubation with primary and secondary antibody was performed at 37°C for 1 hr. The antigen–antibody complexes were detected by a goat anti-human Ig (IgM, IgG, IgA, H + L) peroxidase-conjugated (Southern Biotechnology Association Inc., Birmingham, AL), using the TMB (Vector Laboratories Inc., Burlinghame, CA) as substrate. Optical density was read at 450 nm.

Mouse and Rabbit Immune Sera

Hyperimmune mouse sera were produced by intraperitoneal immunization in CD1 mice (4–8 weeks old) as previously described in Di Bonito et al. [1999], using 10 μg of purified proteins per mouse. Purified proteins (100 μg per animal) were also inoculated intradermally four times, at 3-weeks interval, in albino New Zealand rabbits. The priming immunization was made with each antigen emulsified with Freund’s complete adjuvant (0.5 ml/rabbit). The first and second boosters contained antigen in Freund’s incomplete adjuvant (0.5 ml/rabbit) while the final booster consisted in the blotting using the corresponding recombinant protein as antigen.

Immunocytochemical Assay

Slides containing biochips of SARS-CoV infected and uninfected cells (Euroimmun Italia, Padova, Italy) were treated with 10% fetal calf serum in PBS for 30 min to remove aspecific reaction. Rabbit and mouse anti-sera were serially diluted at 1:100 to 1:25,000 and 1:100 to 1:25,000, respectively. The N and M full-length genes were generated by RT-PCR technology from a 10 μl sample containing heat and gamma-irradiated cell culture supernatant of Vero E6 cells infected with the SARS-CoV isolate Frankfurt 1 [Thiel et al., 2003]. The oligonucleotides used are listed in Table I. The BamHI and PstI sequences were added at the 5′-ends of each forward (FW) and reverse (RV) primer, respectively.

**TABLE I. Primers and Probes Used in RT-PCR Amplification**

| Primer<sup>a</sup> | DNA sequence | Gene target | Nucleotide positions in EMBL no. | AY291315 |
|------------------|--------------|-------------|----------------------------------|----------|
| NQEFW 5′-CGCGGATCCGGGATCTGCTATGTAATGGACC-3′ | Nucleoprotein N | 28120–28137 |
| NQERV 5′-AAAACTGCAAGACTTTATGGCCTTGAGTTGAATC-3′ | Nucleoprotein N | 29388–29369 |
| N1QERV 5′-AAAACTGCAAGGGCAGTTTCATAGTATAGAAGTA-3′ | N1 fragment of N | 28471–28447 |
| N2QEFW 5′-CGCGGATCTCTTCTCTGTTGAGTCTG-3′ | N2 fragment of N | 28447–28470 |
| N2QERV 5′-AAAACTGACGAATTTATGGGCTCCATGATAAGT-3′ | N2 fragment of N | 29116–29113 |
| N3QEFW 5′-CGCGGATCCCACTTATCATGGAGCCATTAAATGTG-3′ | N3 fragment of N | 29116–29139 |
| MQEFW 5′-CGCGGATCCGGGATCTGCTATGTAATGGACC-3′ | Membrane protein M | 26988–26415 |
| MQERV 5′-AAAACTGCAAGCTTACGCTTATGCAAAACG-3′ | Membrane protein M | 27063–27043 |
| M2QEFW 5′-CGCGGATCCGTATTGCTGATTCATCCTG-3′ | M2 fragment of M | 26809–26832 |

<sup>a</sup>BamHI and PstI recognition sequences were added at the 5′-ends of each forward (FW) and reverse (RV) primer, respectively.
1:10,000, respectively in PBS 1% non-fat dry milk and incubated with the cell samples for 1 hr at room temperature. Antigen–antibody complexes were revealed by a detection system using the labeled polymer method (DAKO EnVision™ System, 3-amino-9-ethylcarbazole, Horseradish Peroxidase, Carpinteria, CA). Samples were observed under the light microscope (Leitz, Germany) at a total magnification indicated in the legend of the Figure 4.

RESULTS AND DISCUSSION

N and M structural SARS-CoV proteins were chosen to develop an ELISA test to detect specific antiviral antibodies. The N and M full-length genes were amplified from heat and gamma-irradiated cell culture supernatant of Vero E6 cells infected with the SARS-CoV isolate Frankfurt 1. The amplified cDNAs were cloned in the expression vector pQE-30 (QIAGEN), obtaining the p-N and p-M constructs, respectively. The C-terminus, extra-membrane tail of the M protein (138–222 amino acids) was cloned and sequenced (Fig. 1, panel a). The deduced amino acid sequences of each insert were compared with the N and M sequences of the SARS-CoV Frankfurt 1 isolate revealing a 100% amino acid identity, thus assuring that the cloning methods had no inserted spurious amino acid variations. While the whole protein M could not be generated, probably for its toxicity in E. coli, the recombinant M2 fragment as well as the full length N protein were successfully expressed in E. coli (Fig. 1, panel b). Recombinant proteins were purified and used as coating antigens in ELISA set up to analyze the 6 SARS-positive human sera. Twenty human sera from healthy blood donors and 73 SARS-negative sera from patients affected by acute respiratory syndromes with viral (non-SARS) or bacterial etiology were used as negative controls. All sera from SARS patients showed a strong positive reaction against N and M2 antigen. In contrast, no positive reaction was observed in any serum from non-SARS subjects with the dilution cut-off considered, suggesting a high specificity of the test. Figure 2 particularly shows the titration curves of the 6 SARS serum samples obtained by ELISA with the use of M2 (panel a) and the whole N antigen (panel b). It also shows that the anti-M2 antibody titre was generally lower than the anti-N titre, nonetheless, the anti-M2 response showed particularly low inter-subject variability, as compared to the anti-N response. The mean OD reading values of the six SARS sera tested against either M2 or N antigens, at the dilution of 1:600 and 1:2,000, respectively, are shown in Figure 2, panel c, together with the values of the 93 non-SARS subjects (OD < 0.2). All high-titre ELISA results were confirmed by Western blotting showing the expected immuno-reactive viral protein band (data not shown).

Fig. 1. Panel a: schematic representation of the coding region of SARS-CoV nucleocapsid (N) and membrane (M) proteins. The N1, N2, N3, and M2 fragments of the N and M proteins are represented. Numbers on the bars indicate the first and last amino acids present in the constructs. Panel b: SDS–PAGE analysis of lysates from induced (I) and not induced (NI) E. coli transformed by the expression plasmids containing N, N1, N2, N3, and M and M2 coding sequences. The gel was stained by Coomassie blue. Lanes P show the purified proteins. The molecular mass size markers (in kDa) are reported on the right of the Figure.
Fig. 2. Detection of specific immunoglobulins in SARS-CoV positive human sera using M2 (panel a) and N (panel b) based ELISA. Optical density values are reported in ordinate, dilutions of the sera are reported in abscissa. In N based ELISA, sera dilutions are from 1:2,000 to 1:128,000. In M2 based ELISA, sera dilutions are from 1:600 to 1:22,400. The serum of each SARS patient is indicated by a number (from 1 to 6). Dotted lines indicate SARS-CoV negative human sera. Panel c: comparison between the average OD values of positive and negative SARS-CoV sera obtained from the recombinant based ELISA. The dilutions of sera are of 1:600 and 1:2,000 for M2 and N, respectively. White bars indicate the mean values ± SD of the SARS-CoV negative sera, grey bars indicate the values ± SD of the SARS-CoV positive sera.

Fig. 3. Detection by ELISA of immunoglobulins specific to the N1 (panel a), N2 (b), and N3 (c) fragments of the N protein in SARS-CoV positive human sera. Optical density values are reported in ordinate, dilutions of the sera are reported in abscissa. In N1 and N3 based ELISA, sera dilutions are from 1:600 to 1:22,400. In N2 based ELISA, sera dilutions are from 1:2,000 to 1:128,000. Each human serum is indicated by a number (1–6) on the correspondent line. Dotted lines indicate SARS-CoV negative human sera. Panel d: comparison between the average of the OD values of positive and negative SARS-CoV sera obtained with the N fragments based ELISAs. The dilutions of sera are 1:600 for N1 and N3, and 1:2,000 for N2. White bars indicate the mean values ± SD of 90 SARS-CoV negative sera, grey bars indicate the mean values ± SD of the 6 SARS-CoV referent sera.
animal species, inducing specific antibodies, reactive

All viral proteins showed high immunogenicity in both

proteins were purified and inoculated either in rabbit or

viral antigens. Thus, all the five SARS-CoV recombinant

the recombinant proteins, to be used for the detection of

seria was however observed at 1:1,200 dilution.

no positive reaction with any non-SARS

ELISA at the 1:2,000 dilution and only two out of the 93

positive reaction was observed in the N- and N2-based

the effectiveness of recombinant antigen-based ELISA

tests, in particular with the use of the novel N2 and

M2 antigen for the detection SARS-CoV-antibodies. In

addition, our recombinant proteins have been found to be

useful for the generation of hyperimmune sera to use for

the detection of viral antigens in clinical specimens.

Overall, our results strongly suggest the effectiveness of recombinant antigens M and N in laboratory diagnosis of SARS and invite to further, more extensive investigations to validate our methods.

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REFERENCES

Chan PK, To WK, Ng KC, Lam BK, Ng TK, Chan RC, Wu A, Yu WC, Lee N, Hui DS, Lai ST, Hon EK, Li CK, Sung JJ, Tam JS. 2004. Laboratory diagnosis of SARS. Emerg Infect Dis 10:825–831.

Che XY, Hao W, Qiu LW, Pan YX, Liao ZY, Xu H, Chen JJ, Hou JL, Woo PC, Lau SK, Kwok YY, Huang Z. 2003. Antibody response of patients with severe acute respiratory syndrome (SARS) to nucleocapsid antigen of SARS-associated coronavirus. Di Yi Jun Yi Da Xue Xue Bao 23:637–639.

Chen H, Coote B, Attree S, Hiscox JA. 2003. Evaluation of a nucleoprotein-based enzyme-linked immunoassay for the detection of antibodies against infectious bronchiitis virus. Avian Pathol 32:519–526.

Di Bonito P, Nicoletti L, Mochi S, Accardi L, Marchi A, Giorgi C. 1999. Immunological characterization of Toscana virus proteins. Arch Virol 144:1947–1960.

Donnelly CA, Fisher MC, Fraser C, Ghanii AC, Riley S, Ferguson NM, Anderson RM. 2004. Epidemiological and genetic analysis of severe acute respiratory syndrome. Lancet Infect Dis 4:672–683.

Elia G, Fiormonte G, Prattelli A, Martella V, Camero M, Cirone F, Buonavoglia C. 2003. Recombinant M protein-based ELISA test for detection of antibodies to canine coronavirus. J Virol Methods 109:139–142.

Leung DT, Tam FC, Ma CH, Chan PK, Cheung JL, Niu H, Tam JS, Lim PL. 2004. Antibody response of patients with severe acute
respiratory syndrome (SARS) targets the viral nucleocapsid.
J Infect Dis 190:379–386.
Liu X, Shi Y, Li P, Li L, Yi Y, Ma Q, Cao C. 2004. Profile of antibodies to
the nucleocapsid protein of the severe acute respiratory syndrome
(SARS)-associated coronavirus in probable SARS patients. Clin
Diagn Lab Immunol 11:227–228.
Ndifuna A, Waters AK, Zhou M, Collisson EW. 1998. Recombinant
nucleocapsid protein is potentially an inexpensive, effective
serodiagnostic reagent for infectious bronchitis virus. J Virol
Methods 70:37–44.
Poon LL, Guan Y, Nicholls JM, Yuen KY, Peiris JS. 2004a. The
aetiology, origins, and diagnosis of severe acute respiratory
syndrome. Lancet Infect Dis 4:663–671.
Poon LL, Chan KH, Peiris JS. 2004b. Crouching tiger, hidden dragon:
The laboratory diagnosis of acute respiratory syndrome. Clin Infect
Dis 38:297–299.
Shi Y, Yi Y, Li P, Kuang T, Li L, Dong M, Ma Q, Cao C. 2003. Diagnosis
of severe acute respiratory syndrome (SARS) by detection of SARS
coronavirus nucleocapsid antibodies in an antigen-capturing
enzyme-linked immunosorbent assay. J Clin Microbiol 41:5781–
5782.
Shi Y, Wan Z, Li L, Li P, Li C, Ma Q, Cao C. 2004a. Antibody responses
against SARS-coronavirus and its nucleocapsid in SARS patients.
J Clin Virol 31:66–68.
Shi Y, Yi Y, Li P, Kuang T, Li L, Dong M, Ma Q, Cao C. 2004b. Detection
of severe acute respiratory syndrome (SARS) coronavirus nucleo-
capsid protein in SARS patients by enzyme-linked immunosorbent
assay. J Clin Microbiol 42:2884–2889.
Thiel V, Ivanov KA, Putics A, Hertzig T, Schelle B, Bayer S, Weissbrich
B, Snijder EJ, Rabenau H, Doerr HW, Gorbatenko AE, Ziebuhr J.
2003. Mechanisms and enzymes involved in SARS coronavirus
genome expression. J Gen Virol 84:2305–2315.
Woo PC, Lau SK, Wong BH, Tsioi HW, Fung AM, Chan KH, Tam VK,
Peiris JS, Yuen KY. 2004. Detection of specific antibodies to severe
acute respiratory syndrome (SARS) coronavirus nucleocapsid
protein for serodiagnosis of SARS coronavirus pneumonia. J Clin
Microbiol 42:2306–2309.
Wu HS, Hsieh YC, Su IJ, Lin TH, Chiu SC, Hsu YF, Lin JH, Wang MC,
Chen JY, Hsiao PW, Chang GD, Wang AH, Ting HW, Chou CM,
Huang CJ. 2004. Early detection of antibodies against various
structural proteins of the SARS-associated coronavirus in SARS
patients. J Biomed Sci 11:117–126.