METHOD OF DIAGNOSING SARS CORONA VIRUS INFECTION

CROSS REFERENCE TO RELATED APPLICATION

[0001] The present application claims priority from U.S. Provisional Application No. 60/477,059, filed June 10, 2003, which is herein incorporated by reference.

FIELD OF THE INVENTION

[0002] The present invention relates generally to methods of diagnosing viral infection, and particularly to methods of diagnosing SARS coronavirus infection.

BACKGROUND OF THE INVENTION

[0003] The newly emerged Severe Acute Respiratory Syndrome (SARS) is a serious respiratory illness of global significance. Affected patients can develop symptoms of atypical pneumonia and early and accurate differentiation of SARS from atypical pneumonia is critical for successful management of this highly contagious disease.

[0004] The highly contagious nature of SARS, combined with an eminent mortality rate can be disruptive and costly in an increasingly globalized world. This was promptly recognized by the World Health Organization (WHO) in 2003 when the SARS epidemic spread beyond its place of origin in Guangdong Province, China, and a global alert was declared for the first time in WHO history. The outbreaks affected over 8,098 people and spread to more than 29 countries and regions in a short period of 6 months, with a mortality rate of up to 15%. Obviously, this disease is not a limited problem but one with profound consequences affecting sectors beyond public health care. As new cases appear to be re-emerging as of January 2004, the urgency remains for prompt identification and isolation of infected patients.

[0005] In many countries, to reduce the risk of contact with people who may have been exposed to the SARS-causing virus, strict quarantine orders are served to those who have travelled to SARS-affected countries, those who had been in direct contact

with SARS patients, and those with temperatures exceeding 38°C. Early diagnoses of the disease during the early phase of infection could avoid unnecessary quarantines, reduce the stress to those concerned, and help doctors to decide on appropriate medical action and/or treatment. It is therefore vital to identify SARS patients as early as possible, with certainty and accuracy.

[0006] A novel coronavirus was identified as the etiological agent of SARS (1- 4). Coronaviruses are enveloped viruses that contain a single-stranded, positive-sense RNA genome of 27.6 to 31 kb. Analyses of the nucleotide sequence of the novel SARS coronavirus (SARS CoV) showed that the viral genome is nearly 30 kb in length (5, 6) and contains 14 potential open reading frames (ORFs) (5). However, more remains to be learned in terms of how to detect, diagnose, and treat the disease. Prompt identification and isolation of infected patients remains of paramount importance for disease control, since no drug or vaccine is available for this disease.

[0007] Initially, when SARS was first identified, initial disease management relied on travel and contact history and presentation of symptoms. Subsequently, with the identification of the SARS-CoV genome, several diagnostic tests based on the detection of viral RNA sequences by use of PCR have been designed and are now available. WHO has, based on these methods, revised its criteria for case definition.

[0008] However, existing protocols for PCR are not without their limitations. Such tests, although sensitive, have inherent problems: scientists and clinicians around the world are unsure what types of samples (respiratory samples, saliva, stool, blood, or conjunctival fluid) from patients give the most reproducible RNA preparations; RNA extraction protocols are not straightforward, and if not done well, may produce RNA preparations that are not useful for the reverse transcription step that converts viral RNA to DNA; and the whole process of extraction, reverse transcription, and PCR can be time-consuming if confirmatory tests have to be done with several pairs of primers. In addition, false positives are possible with amplification methods, as was observed in August 2003 in Canada, when some patients infected with other human coronaviruses initially tested positive for SARS by a PCR method. Contamination in PCR laboratories is always a concern, which in the case of SARS could lead to unnecessary quarantines.

[0009] Results obtained in Singapore showed that the positive predictive values (PPVs) provided by the PCR diagnostic method varied from 56.7 to 83.8% when different clinical specimens were used (A. E. Ling, "SARS—the Singapore laboratory experience," presented at the WHO Conference on SARS Research, Singapore, Republic of Singapore, 19 June 2003).

[0010] In a very recent report (7), the reverse transcription-PCR (RT-PCR) protocols of two WHO SARS network laboratories were evaluated, and the findings confirmed similar shortcomings of RT-PCR. Therefore, the existing PCR cannot rule out the presence of the SARS virus when a negative result is obtained; neither can it exclude the possibility of false detection due to laboratory contamination (6, 8, 9).

[0011] Diagnostic methods involving indirect immunofluorescence assay (IFA), classic tissue culture for viral isolation, and electronic microscopy of the cell culture for diagnosis generally tend to be either time-consuming or technically very demanding. Alternative approaches are therefore critical for efficient management of the disease.

[0012] Although the identification of the novel coronavirus, SARS-CoV as the etiological agent for SARS made it possible for various tests to be developed, providing tools for laboratory diagnosis stays a priority as suggested by WHO. To date, there is still no standardized test for diagnosing SARS regardless whether tests are antigen-based or antibody-based. Testing newly developed kits with standardized panels or by a trial using large numbers of clinical specimens will provide useful information addressing the shortcoming.

SUMMARY OF THE INVENTION

[0013] In one aspect, the invention provides a method for detecting the presence or absence of antibody to SARS coronavirus (CoV), the method comprising the step of contacting a SARS CoV antigen with an antibody-containing sample, for a time and under conditions sufficient for the antibody to form a complex with the antigen, wherein the antigen comprises a protein selected from the group consisting of S, M, E, N, U274 and an immunogenic fragment thereof, followed by the step of detecting the complex by contacting the complex with an anti-human IgA antibody or an anti-human IgM antibody, and wherein detection of the specific binding between the antibody and the protein complex indicates that the sample contains antibody specific to SARS CoV. The method may be used to test whether a human is infected by, or has been exposed to, SARS CoV, wherein the

antibody-containing sample is from the human being tested, and wherein specific binding between the antibody and the protein indicates that the human is infected by or has been exposed to SARS CoV.

[0014] The invention also provides commercial packages. In one aspect, there is provided a commercial package for detecting the presence or absence of antibody to SARS coronavirus (CoV) in a sample, comprising a SARS CoV antigen comprising a protein selected from the group consisting of S, M, E, N, U274, and an immunogenic fragment thereof; and means for detecting a complex between the antibody from the sample and the antigen comprising an anti-human IgA antibody or an anti-human IgM antibody.

[0015] In another aspect, there is provided a commercial package for testing whether a human is infected by or has been exposed to SARS coronavirus (CoV), comprising a SARS CoV antigen comprising a protein selected from the group consisting of S, M, E, N, U274, and an immunogenic fragment thereof; and means for detecting a complex between the antigen and an antibody against the antigen comprising an anti-human IgA antibody or an anti-human IgM antibody, wherein the sample is an antibody-containing sample of the human being tested.

[0016] In another aspect, the invention provides an isolated antibody directed against an antigenic SARS CoV protein, wherein the antigenic SARS CoV protein is S, M, E, N or U274 protein or an antigenic fragment thereof.

[0017] The antibodies of the invention may be used to detect the presence of S, M, E, N or U274 protein of SARS CoV in a sample. Thus, in another aspect, the invention provides a method of detecting the presence or absence of S, M, E, N or U274 SARS CoV protein, the method comprising contacting an antibody directed against a protein selected from the group consisting of S, M, E, N and U274, with an antigen-containing sample, for a time and under conditions sufficient for the antibody to form a complex with an antigen followed by the step of detecting the complex by contacting the complex with an anti-human IgA antibody or an anti-human IgM antibody; and wherein detection of the specific binding between the antibody and the antigen complex indicates that the sample contains an antigen that comprises S, M, E, N, U274 or an immunogenic fragment thereof.

[0018] Other aspects and features of the present invention will become apparent to those of ordinary skill in the art upon review of the following description of specific embodiments of the invention in conjunction with the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] In the figures, which illustrate, by way of example only, embodiments of the present invention,

[0020] **Figure 1** is a schematic depiction of the structural organization and expression of ORFs corresponding to structural proteins S, M, N, and E and unique proteins (UX), where "X" denotes the number of amino acids encoded by the respective ORF; corresponding annotated ORFs 1 to 14 are indicated;

[0021] **Figure 2** is a Western blot analysis demonstrating the presence of antibodies against the various SARS-CoV viral proteins in patient sera;

[0022] **Figure 3** depicts bacterially expressed GST-U274, GST-U122, GST-N, and GST proteins analyzed by SDS-PAGE and stained with Coomassie brilliant blue R-250 (Bio-Rad);

[0023] **Figure 4** illustrates detection of bacterially expressed viral proteins by Western blot analysis with anti-GST antibody, antibodies in a control serum, or antibodies in six convalescent-phase sera;

[0024] **Figure 5** illustrates detection of N and U274 by Western blot using sera from patients during early and late phases of infection as primary antibody and anti-human IgG as secondary antibody;

[0025] **Figure 6** is a Western blot using some of the samples as depicted in Figure 5, using anti-human IgG, anti-human IgA, or anti-human IgM antibody as secondary antibody;

[0026] **Figure 7** illustrates detection of anti-S antibodies in patient sera by an immunofluorescence method utilizing CHO cells stably expressing the S protein;

[0027] **Figure 8** is a photograph of examples of the assembled rapid immunochromatographic test devices with their separators (transparent tabs) at the "removed" (after assay) position, either after an assay with a sample from an infected patient (left), or after an assay with a sample from a healthy control (right), showing

lines for the control, Gst-N, and Gst-U274, from top to bottom respectively;

[0028] **Figure 9** is a scatter chart of OD values obtained with sera from both SARS patients and healthy controls tested for IgG antibody to SARS CoV, using an ELISA assay;

[0029] **Figure 10** is a line graph showing titration curves obtained with the ELISA assay using serial dilutions of seven SARS patient samples;

[0030] **Figure 11** illustrates the correlation between the ELISA and the rapid immunochromatography test when comparing the dilutions at which reactivity end points were obtained with seven SARS patient samples;

[0031] **Figure 12** illustrates the distribution of percentage detection rates of the ELISA assay and the rapid immunochromatography assay in relation to sample immunofluorescence titers;

[0032] **Figure 13** illustrates Western immunoblot pattern of immunoreactive proteins of SARS-CoV;

[0033] **Figure 14** is a Western blot demonstrating the location of the immunoreactive proteins of SARS-CoV as identified with antibodies raised against specific recombinant proteins (1: SARS-positive control; 2: mouse anti-spike protein antiserum; 3: mouse anti-nucleocapsid protein antiserum; 4: mouse anti-matrix protein antiserum; 5: rabbit anti-envelop protein antiserum); and

[0034] **Figure 15** shows representative immunoreactive patterns of the SARS Western immunoblot with serum samples (A: SARS patients; B: healthy controls; C: non-SARS fever patient controls; D: non-SARS respiratory disease controls; D: false positive identified by ELISA).

DETAILED DESCRIPTION OF EMBODIMENTS

[0035] The sequence of SARS-CoV reveals ORFs for four structural proteins, i.e., spike (S), membrane (M), envelope (E), and nucleocapsid (N), which are conserved in all coronaviruses (5, 6, 10, 11). The S protein plays essential roles in mediating

receptor binding and internalization of the virus and is one of the major antigens of the virus. The M and E proteins are essential for virion assembly, and the N protein binds to the viral genome to form the nucleocapsid.

[0036] Besides these four common structural proteins, there are several unique ORFs predicted from the SARS-CoV sequence that show no significant sequence homology to viral proteins of other coronaviruses. Whether these ORFs express proteins that serve a function in the viral replication cycle are yet to be determined.

[0037] The present invention relates to the discovery that the S, M, E and N structural proteins of SARS CoV, as well as one of the proteins of unknown function having a predicted length of 274 amino acids (referred to herein as U274), are antigenic and may be used to detect the presence of anti-SARS CoV antibodies in a patient, indicating the patient is infected with SARS CoV, or has been exposed to SARS CoV.

[0038] Therefore, in one aspect there is provided a method of diagnosing SARS CoV infection in a patient, using the antigenic SARS CoV proteins S, M, E, N and U274. One or more of the antigenic proteins is expressed, and is contacted with a biological sample from a patient whom is to be tested for SARS CoV infection. If the patient is infected with SARS CoV or has been exposed to SARS CoV, and has generated antibodies against the virus, antibodies in the sample directed against the particular SARS CoV antigenic protein being used for diagnosis will bind to the protein. A secondary antibody directed against the patient-generated antibodies and conjugated to a detection molecule may be used to detect the patient-generated antibodies.

[0039] As used herein, the term "antigenic protein" of SARS CoV refers to one or more of the SARS CoV S, M, E, N or U274 protein. The term "immunogenic fragment thereof" or "antigenic fragment thereof" refers to a portion of the SARS CoV S, M, E, N or U274 protein which is immunogenic or antigenic, meaning that the protein fragment will contain one or more epitopes, thereby being capable of eliciting an immune response in a patient.

[0040] It is an accepted practice in the field of immunology to use fragments and

variants of protein as immunogens, as all that is required to induce an immune response to a protein is a small (*e.g.*, 8 to 10 amino acid) immunogenic region of the protein. Various short synthetic peptides corresponding to surface-exposed antigens of pathogens have been shown to be effective antigens against their respective pathogens, *e.g.* an 11 residue peptide of murine mammary tumor virus (Casey & Davidson, Nucl. Acid Res. (1977) 4:1539), a 16-residue peptide of Semliki Forest virus (Snijders *et al.*, 1991. J. Gen. Virol. 72:557-565), and two overlapping peptides of 15 residues each from canine parvovirus (Langeveld *et al.*, Vaccine 12(15):1473-1480, 1994).

[0041] Accordingly, it will be readily apparent to one skilled in the art, having read the present description, that partial sequences of any one of S, M, E, N or U274 are inherent to the full-length proteins and are taught by the present invention. Such polypeptide fragments preferably are at least 12 amino acids in length. Advantageously, they are at least 15 amino acids, preferably at least 20, 25, 30, 35, 40, 45, 50 amino acids, more preferably at least 55, 60, 65, 70, 75 amino acids, and most preferably at least 80, 85, 90, 95, 100 amino acids in length.

[0042] As used herein, "homologous" or "homolog" refers to a protein sequence that has substantial correspondence with the amino acid sequence of S, M, E, N or U274, or a fragment thereof. The homolog may be at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 87%, 90%, 93%, 96% and 99% homologous to S, M, E, N or U274, or a fragment thereof. Homology is measured using sequence analysis software such as Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705. Amino acid sequences are aligned to maximize identity. Gaps may be artificially introduced into the sequence to attain proper alignment. Once the optimal alignment has been set up, the degree of homology is established by recording all of the positions in which the amino acids of both sequences are identical, relative to the total number of positions.

[0043] Polynucleotides of 30 to 600 nucleotides, as applicable, encoding partial sequences of, or sequences homologous to, or partial sequences homologous to, any one of S, M, E, N and U274 are retrieved by PCR amplification using the parameters outlined above and using primers matching the sequences upstream and downstream

of the 5' and 3' ends of the fragment to be amplified. The template polynucleotide for such amplification is either the full length polynucleotide that encodes full-length S, M, E, N or U274, or a polynucleotide contained in a mixture of polynucleotides such as a DNA or RNA library. As an alternative method for retrieving the partial sequences, screening hybridization is carried out under conditions described above and using the formula for calculating Tm. Where fragments of 30 to 600 nucleotides are to be retrieved, the calculated Tm is corrected by subtracting (600/polynucleotide size in base pairs) and the stringency conditions are defined by a hybridization temperature that is 5 to 10°C below Tm. Where oligonucleotides shorter than 20-30 bases are to be obtained, the formula for calculating the Tm is as follows: Tm = 4 x (G+C) + 2 (A+T). For example, an 18 nucleotide fragment of 50% G+C would have an approximate Tm of 54°C. Short peptides that are fragments of any one of S, M, E, N or U274 or its homologous sequences, are obtained directly by chemical synthesis.

[0044] Useful polypeptide derivatives, *e.g.*, polypeptide fragments, are designed using computer-assisted analysis of amino acid sequences. This would identify probable surface-exposed, antigenic regions (Hughes *et al.*, 1992. Infect. Immun. 60(9):3497). Analysis of amino acid sequences contained in any one of S, M, E, N or U274, based on the product of flexibility and hydrophobicity propensities using the program SEQSEE (Wishart DS, *et al.* "SEQSEE: a comprehensive program suite for protein sequence analysis." *Comput Appl Biosci.* 1994 Apr;10(2):121-32), can be used to reveal potential B- and T-cell epitopes which may be used as a basis for selecting useful immunogenic fragments and variants. This analysis uses a reasonable combination of external surface features that is likely to be recognized by antibodies. Probable T-cell epitopes for HLA-A0201 MHC subclass may be revealed by an algorithm that emulates an approach developed at the NIH (Parker KC, *et al.* "Peptide binding to MHC class I molecules: implications for antigenic peptide prediction." *Immunol Res* 1995;14(1):34-57).

[0045] Epitopes which induce a protective T cell-dependent immune response are present throughout the length of the polypeptide. However, some epitopes may be masked by secondary and tertiary structures of the polypeptide. To reveal such masked epitopes large internal deletions are created which remove much of the original protein structure and expose the masked epitopes. Such internal deletions

sometimes effect the additional advantage of removing immunodominant regions of high variability among strains.

[0046] Polynucleotides encoding polypeptide fragments and polypeptides having large internal deletions are constructed using standard methods (Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons Inc., 1994). Such methods include standard PCR, inverse PCR, restriction enzyme treatment of cloned DNA molecules, or the method of Kunkel *et al.* (Kunkel *et al.* Proc. Natl. Acad. Sci. USA (1985) 82:448). Components for these methods and instructions for their use are readily available from various commercial sources such as Stratagene.

[0047] The full-length antigenic protein or proteins may be used in the diagnosis method. However, it is preferred to use an antigenic fragment of the relevant SARS CoV antigenic protein that is not highly conserved among coronaviruses, so as to minimize the chance of cross-reactivity with patient-generated antibodies directed against other coronaviruses. As well, it may be advantageous to delete portions of the relevant SARS CoV antigenic protein that are predicted to be transmembrane domains, or to use only a soluble antigenic fragment of the relevant protein, for ease of expression and handling of the protein, as will be understood by a skilled person.

[0048] Interestingly, unlike some coronaviruses in which the M protein is most abundant, N appears to be the most abundant protein in SARS-CoV (6). The results presented herein indicate that N generates a strong immunoreaction.

[0049] Without intending to be bound by theory, it is possible that N may be released from SARS CoV or from infected patient cells into the circulation at some stage of infection, or possibly that N may be presented by antigen-presenting cells for cytotoxic killing of infected cells. Furthermore, it is possible that N may contribute to the humoral immune response protecting patients against SARS.

[0050] For the N protein, when the sequences of 18 SARS isolates deposited in the GenBank database were compared, only two of them showed a difference, at one amino acid, from that of the Singapore isolate (SIN2774) used for the clones described in the Examples below (13). Therefore, the N protein does not appear to undergo rapid mutation, which is another advantage for its use in the present method.

[0051] The presence of antibodies directed against N can be detected in patients as early as 2 days post-infection, as well as later in infection, for example, 16 days post-infection. The early elicitation of immune response, in combination with an extremely strong immune response make N extremely useful in diagnosing SARS CoV infection.

[0052] Thus, in various preferred embodiments, the protein used to detect the presence of anti-SARS CoV antibodies in a patient is N or an antigenic fragment thereof. N may be full-length N, or it may be N (Δ111-118), which refers to N having amino acids 111 to 118 deleted. These amino acids correspond to the sequence FYYLGTGP, a highly conserved sequence found in the N homologue of coronaviruses. An immunogenic fragment of N may be used, for example a fragment comprising or consisting of amino acids 69 to 422, 120 to 422, or 121 to 422.

[0053] S forms the petal-shaped spikes found on the surfaces of coronaviruses (10, 11). This protein is also highly immunogenic. S is a large protein, with a number of transmembrane regions, and is highly glycosylated. The S protein is predicted to contain hypervariable regions, as these regions appear in S proteins of other coronaviruses (10, 11). It may be preferred to use an extracellular region of S that does not contain a hypervariable region, to ensure ease of handling and to increase the likelihood of a properly positive diagnosis for patients infected with SARS CoV.

[0054] Thus, in certain embodiments, full-length S may be used, or for example, a fragment of S comprising or consisting of amino acids 460 to 480 may be used.

[0055] The majority of the patients tested to date have antibodies against the C-terminal end of U274 (U274 corresponding to ORF 3, as annotated in reference 5, and X1 in reference 6). The immunoreactivity of U274 indicates that this novel and unique viral protein is expressed in the virus and is likely to be a protein involved in the biogenesis of SARS-CoV. Moreover, a potential transcription regulating sequence was found upstream of the U274 coding sequence, suggesting that it is the first ORF for one of the major subgenomic RNAs of SARS-CoV-infected cells (5, 6). U274 does not share significant homology with any protein in the database, but it appears to have a similar topology to that of M, with three transmembrane regions and a large internal C-terminal domain. Since it easier to express and purify soluble

fragments of proteins, it may be preferred, when using U274 in the present method, to use a fragment that does not include the transmembrane regions of U274.

[0056] As well, U274 does tend to exhibit some low level sequence variation in viral isolates, with five isolates of those studied having U274 sequences showing one amino acid substitution (when compared to SIN2774) and one isolate having a U274 sequence showing two amino acid substitutions (14).

[0057] Thus, in certain embodiments, the antigenic SARS CoV protein used may be full-length U274, or it may be an antigenic fragment of U274 comprising or consisting of amino acids 134 to 274.

[0058] Of patients tested to date, not all possessed antibodies directed against the M and E proteins of SARS CoV. Therefore, these proteins, although antigenic, may be less useful in the present diagnostic method, since it seems that they do not consistently elicit an immune response in infected individuals. In various embodiments, the antigenic SARS CoV protein used may be full-length M, or it may be an antigenic fragment of M comprising or consisting of amino acids 98 to 121. In other embodiments, the antigenic SARS CoV protein used may be full-length E, or it may be an antigenic fragment of E comprising or consisting of amino acids 38 to 76.

[0059] In the present method, the antigenic protein or proteins are first expressed, and may be expressed using methods known in the art. "Expressing" a protein refers to the synthesis of a protein or polypeptide by the translation of an RNA template, usually an mRNA, which encodes the protein or polypeptide and may include a transcription step in which an RNA template is transcribed by an RNA polymerase enzyme from a DNA template. The protein may be expressed within in any expression system, such as a cell, or within a cell-free system.

[0060] For example, the proteins may be expressed in a prokaryotic expression system, for example in bacteria such as *Escherichia coli*, or in a eukaryotic expression system, for example, in yeast including *Saccharomyces cerevisiae* or *Pichia pastoris*, in mammalian cells including COS1, COS7, CHO, NIH3T3, or JEG3 cells, in insect cells including *Spodoptera frugiperda* (SF9) cells, or in plant cells.

[0061] The proteins may be expressed in a cell-free expression system, and such a

system will include the reagents necessary to effect expression of the protein, including ribosomes, tRNAs, amino acids, including amino acyl tRNAs, RNA template, and may further include DNA template, RNA polymerase, ribonucleotides, and any necessary cofactors, buffering agents and salts that are required for enzymatic activity, and may include a cell lysate.

[0062] A skilled person will understand how to express the desired protein or protein fragment in an appropriate expression system. For a protein that is not post-translationally modified and is expected to be soluble, a bacterial expression system may be preferred. However, for large proteins, proteins that are post-translationally modified, or proteins that are require mRNA splicing, a eukaryotic system, for example a mammalian system, may be preferred.

[0063] Typically, a gene encoding the antigenic protein or protein fragment will be cloned into an expression vector that is compatible with the expression system chosen to express the antigenic protein, using standard techniques known in the art. The expression vector will have the necessary promoter and genetic signals required to effect expression in the chosen system. For example, the expression vector may comprise a gene encoding the protein operably linked to a promoter that is compatible with the particular expression system, and may be a plasmid. For example, the cell may be an *E. coli* cell, and the expression vector may contain a gene encoding the antigenic SARS CoV protein of interest operably linked to all of the necessary regulatory sequences such that the gene is transcribed and the RNA is translated by the *E. coli* cellular machinery. The expression of the gene encoding the protein of interest may be driven by an inducible promoter such that the expression within the cell may be controlled as desired, so as to maximize expression, for example by synchronizing protein expression with logarithmic growth phase of the cell culture.

[0064] For ease of purification and use, the proteins may be expressed as a fusion protein. A fusion protein is a protein or polypeptide that contains an antigenic SARS CoV protein fused at the N- or C-terminal end to a second polypeptide. A simple way to obtain such a fusion polypeptide is by translation of an in-frame fusion of the polynucleotide sequences, *i.e.*, a hybrid gene. The hybrid gene encoding the fusion polypeptide is inserted into an expression vector which is used to transform or transfect a host cell. Alternatively, the polynucleotide sequence encoding the

polypeptide or polypeptide derivative is inserted into an expression vector in which the polynucleotide encoding the second peptide is already present. The second peptide may be an affinity peptide or protein sequence that binds to a ligand or functional group, to assist in purification. For example, the second peptide may be a six-histidine sequence which binds to Ni^{+2} ions, or it may be glutathione-S-transferase (GST) which binds to glutathione.

[0065] Once expressed, the antigenic protein may be purified, using standard purification techniques, such as affinity column chromatography, for example, using a chromatography column having bound glutathione to purify a GST fusion protein. Using known affinity chromatography methods, a fusion protein containing the antigenic protein may be purified to a high degree, which results in a diagnosis method having better sensitivity and specificity when compared with the use of coarse viral lysates for serological assays, since the presence of antibodies against cellular components in viral lysates can result in false positivity.

[0066] The expressed antigenic protein is used to determine if the patient has generated antibodies directed against SARS CoV by contacting the protein with a sample from the patient. Upon contact, the patient-generated antibodies in the patient sample that can recognize the antigenic SARS CoV protein will bind to the antigenic protein, forming an antigen-primary antibody complex.

[0067] "Specific binding" between an antibody and an antigen, or "specificity" of an antibody for an antigen, refers to the ability of the antibody to recognise and selectively bind to a particular epitope contained within the antigen. Such binding is determined by complementarity between the antigen-binding sites of the antibody and the epitope of the antigen. Complementarity refers to a reciprocal pairing of the spatial arrangement of chemical moieties and charges within the binding sites of each of the antibody and antigen.

[0068] The sample is any sample that contains patient-generated antibodies, either as cell-surface bound antibodies or as circulating antibodies. The sample may be blood, plasma, or serum. The sample is taken from any patient suspected of being infected with SARS, and may be taken between 2 and 60 days post-infection, between 16 and 60 days post-infection or between 2 and 11 days post-infection. The term

"days post-infection" refers to the length of time in days following the time at which it is suspected that the patient came into contact with or contracted SARS CoV.

[0069] The contacting of an antigenic SARS CoV protein is done in the context of an immunoassay, such as ELISA (enzyme-linked immunosorbent assay), for detection of antibodies to S, M, E, N or U274. The antigenic protein may be immobilized on a solid support prior to contacting with the patient sample. Alternatively, the antigenic protein may be expressed in a cell or at a cell surface. Immunoassay techniques are generally known to a skilled person and include ELISAs, Western immunoblots, immunofluorescence assays and immunochromatography assays.

[0070] In order to remain within the detection limits of the immunoassay, it may be necessary to dilute the patient sample. Serial dilutions of the sample may be performed in order to determine the optimal dilution for a sample within a particular testing format. For example, the patient sample may be diluted in a suitable buffer in a ratio of sample to total volume of about 1:10, about 1:20, about 1:40, about 1:80, about 1:160, about 1:320, about 1:640.

[0071] The term "immunoassay" as used herein refers to an analytical method that uses the ability of an antibody to bind a particular antigen as the means for determining the presence of the antigen or antibody. An antibody-capture immunoassay is an assay that provides an antigen which is used to detect antibodies against a particular pathogen in a biological sample of a test subject. In general, the antigen is immobilized on a support and is capable of binding an antibody in a biological sample. The antibody is provided by the biological sample. In a variation of the antibody-capture assay the antigen is mixed with the antibody in the biological sample and the antigen-antibody complex thus formed is captured by a second antibody against the antigen or antibody or both in the antigen-antibody complex which is immobilized on a support. Alternatively, the formation of the antigen-antibody complex is measured in solution.

[0072] It is contemplated that a range of immunoassay formats be encompassed by this definition, including but not limited to direct immunoassays, indirect immunoassays, and "sandwich" immunoassays. A particularly preferred format is a sandwich enzyme-linked immunosorbent assay (ELISA). However, it is not intended

that the present invention be limited to this format. It is contemplated that other formats, including radioimmunoassays (RIA), immunofluorescent assays (IFA), and other assay formats, including, but not limited to, variations on the ELISA method will be useful in the method of the present invention. Thus, other antigen-antibody reaction formats may be used in the present invention, including but not limited to "flocculation" (ie., a colloidal suspension produced upon the formation of antigen-antibody complexes), "agglutination" (i.e., clumping of cells or other substances upon exposure to antibody), "particle agglutination" (i.e., clumping of particles coated with antigen in the presence of antibody or the clumping of particles coated with antibody in the presence of antigen); "complement fixation" (ie., the use of complement in an antibody-antigen reaction method), and other methods commonly used in serology, immunology, immunocytochemistry, histochemistry, and related fields.

[0073] Detection of an antibody-antigen complex can be performed by several methods. The mobile antigen may be prepared with a label such as biotin, an enzyme, a fluorescent marker, or radioactivity, and may be detected directly using this label. Alternatively, a labelled "secondary antibody" or "reporter antibody" which recognizes the primary antibody may be added, forming a complex comprised of antigen-antibody-antibody. Again, appropriate reporter reagents are then added to detect the labelled antibody. Any number of additional antibodies may be added as desired. These antibodies may also be labelled with a marker, including, but not limited to an enzyme, fluorescent marker, radioactivity, or a heavy metal complex. Either the antigen or the antibody (primary or secondary) may be immobilized on a solid support, but the labelled component cannot be immobilized because the detectable signal is precluded from being a measure of binding.

[0074] As used herein, the term "reporter reagent" is used in reference to compounds which are capable of detecting the presence of antibody bound to antigen. For example, a reporter reagent may be a calorimetric substance which is attached to an enzymatic substrate. Upon binding of antibody and antigen, the enzyme acts on its substrate and causes the production of a colour. Other reporter reagents include, but are not limited to fluorogenic and radioactive compounds or molecules. This definition also encompasses the use of biotin and avidin-based compounds (e.g., including compounds but not limited to neutravidin and streptavidin) as part of the

detection system. In one embodiment of the present invention, biotinylated antibodies may be used in the present invention in conjunction with avidin-coated solid support.

[0075] As used herein, the term "solid support" is used in reference to any solid material to which reagents such as antibodies, antigens, and other compounds may be attached. For example, in the ELISA method, the wells of microtiter plates often provide solid supports. Other examples of solid supports include microscope slides, coverslips, beads, particles, cell culture flasks, as well as many other items.

[0076] A kit, or a commercial package, for detecting antibodies to S, M, E, N or U274 generally comprises, in an amount sufficient for at least one assay, an antigenic SARS CoV protein and means for detecting a complex between the antigenic SARS CoV protein and the antibody from the patient sample, as packaged immunochemical reagents. Instructions for use of a packaged immunochemical reagent are also typically included.

[0077] As used herein, the term "packaged" can refer to the use of a solid matrix or material such as glass, plastic, paper, fiber, foil and the like capable of holding within fixed limits an antibody of this invention. Thus, for example, a package can be a glass vial used to contain milligram quantities of a contemplated antigen or it can be a microtiter plate well to which microgram quantities of a contemplated antigen has been operatively affixed. Alternatively, a package could include antigen-coated microparticles entrapped within a porous membrane or embedded in a test strip or dipstick, etc. Alternatively, the antigen can be directly coated onto a membrane, test strip or dipstick, etc. which contacts the sample fluid. Many other possibilities exist and will be readily recognized by those skilled in this art.

[0078] Instructions for use typically include a tangible expression describing the reagent concentration or at least one assay method parameter such as the relative amounts of reagent and sample to be admixed, maintenance time periods for reagent/sample admixtures, temperature, buffer conditions and the like.

[0079] In preferred embodiments, a kit of the present invention further includes a label or indicating means capable of signaling the formation of a complex between the antigenic SARS CoV protein and the antibody.

[0080] As used herein, the terms "label" or "labelling agent" and means for detecting the antibody-antigen complex ("indicating means") refer to molecules that are either directly or indirectly involved in the production of a detectable signal to indicate the presence of a complex. Any label or indicating means can be linked to or incorporated in an expressed protein, peptide, or antibody molecule that is part of the present invention, or used separately, and those atoms or molecules can be used alone or in conjunction with additional reagents. Such labels are themselves well known in clinical diagnostic chemistry. For example, the label or indicating means may be an enzyme that cleaves a reagent to produce a coloured molecule, a coloured molecule, a fluorescent molecule, a radioactive molecule, a chemiluminescent molecule or a heavy metal complex. The precise method of detecting the signal produced by the label or indicating means will depend on the label or indicating means used and the particular immunoassay technique used, as will be understood by a skilled person.

[0081] The label or indicating means can be a fluorescent labeling agent that chemically binds to antibodies or antigens without denaturing them to form a fluorochrome (dye) that is a useful immunofluorescent tracer. Suitable fluorescent labeling agents are fluorochromes such as fluorescein isocyanate (FIC), fluorescein isothiocyante (FITC), 5-dimethylamine-1-natpthalenesulfonyl chloride (DANSC), tetramethylrhodamine isothiocyanate (TRITC), lissamine, rhodamine 8200 sulphonyl chloride (RB 200 SC) and the like.

[0082] In preferred embodiments, the label or indicating means is an enzyme, such as horseradish peroxidase (HRP), glucose oxidase, or the like. In such cases where the principle indicating group is an enzyme such as HRP or glucose oxidase, additional reagents are required to indicate that a receptor-ligand complex (immunoreactant) has formed. Such additional reagents for HRP include hydrogen peroxide and an oxidation dye precursor such as diaminobenzidine or tetramethylbenzidine. An additional reagent useful with glucose oxidase is 2,2,-azino-di-(3-ethyl-benzthiazoline-G-sulfonic acid) (ABTS).

[0083] Radioactive elements are also useful labeling agents and are used illustratively herein. An exemplary radiolabeling agent is a radioactive element that produces gamma ray emissions. Elements which themselves emit gamma rays, such as ^{124}I, ^{125}I, ^{128}I, ^{132}I and ^{51}Cr represent one class of gamma ray emission-producing

radioactive element indicating groups. Particularly preferred is ^{125}I. Another group of useful labeling means are those elements such as ^{11}C, ^{18}F, ^{15}O and ^{13}N which themselves emit positrons. Also useful is a beta emitter, such as ^{111}indium or ^{3}H.

[0084] The linking of labels, i.e., labeling of peptides and proteins is well known in the art. For instance, monoclonal antibodies produced by a hybridoma can be labeled by metabolic incorporation of radioisotope-containing amino acids provided as a component in the culture medium. The techniques of protein conjugation or coupling through activated functional groups are particularly applicable.

[0085] The methods and kits of this invention can also include, preferably as a separate package, a "specific binding agent," which is capable of selectively binding an antibody or antigen of this invention or a complex containing such a species, but is not itself antigen or antibody of this invention. Exemplary specific binding agents are second antibody molecules, e.g. anti-human antibodies, complement proteins or fragments thereof, S. aureus protein A, and the like. Preferably the specific binding agent binds the antibody or antigen when it is present as part of a complex.

[0086] In preferred embodiments, the specific binding agent is labeled. However, when the method or kit includes a specific binding agent that is not labeled, the agent is typically used as an amplifying means or reagent to amplify the signal. In these embodiments, the labeled specific binding agent is capable of specifically binding the amplifying means when the amplifying means is bound to a complex.

[0087] The kits of the present invention can be used in an "ELISA" format to detect the quantity of anti-S, M, E, N or U274 antibody in a fluid sample or extract. "ELISA" refers to an enzyme linked immunosorbent assay such as those discussed above, which employ an antibody or antigen bound to a solid phase and an enzyme-antigen or enzyme-antibody conjugate to detect and quantify the amount of an antigen present in a sample.

[0088] In a number of embodiments, an antigenic SARS CoV protein can be affixed to a solid matrix to form a solid support. A reagent is typically affixed to a solid matrix by adsorption from an aqueous medium although other modes of affixation applicable to proteins and peptides well known to those skilled in the art,

can be used.

[0089] Useful solid matrices are also well known in the art. Such materials are water insoluble and include the crosslinked dextran available under the trademark SEPHADEX from Pharmacia Fine Chemicals (Piscataway, N.J.); agarose; polystyrene beads about 1 micron to about 5 millimeters in diameter polyvinyl chloride, polystyrene, crosslinked polyacrylamide, nitrocellulose- or nylon-based webs such as sheets, strips or paddles; or tubes, plates or the wells of a microtiter plate such as those made from polystyrene or polyvinylchloride.

[0090] The immunoreagents of any diagnostic system described herein can be provided in solution, as a liquid dispersion or as a substantially dry powder, e.g., in lyophilized form. Where the indicating means is an enzyme, the enzyme's substrate can also be provided in a separate package. A solid support such as the above-described microtiter plate and one or more buffers can also be included as separately packaged elements in the diagnostic assay systems of this invention.

[0091] Preferably, the antigenic SARS CoV protein is coated or adsorbed on to the surface of a substrate. A sample of interest is contacted with the antigenic SARS CoV protein and any antibodies to the antigen which may be present in the sample bind to the antigen. Anti-human antibodies are contacted with the antigen/sample and bind to the antibodies that are bound to the antigenic SARS CoV protein.

[0092] The secondary antibody used may be generated in any animal that is not the same species as the patient being tested. Thus mouse anti-human antibodies may be used, or the secondary antibody may be rabbit anti-human or goat anti-human. The anti-human antibodies may be, for example, directed against human IgG, IgM or IgA antibodies.

[0093] The anti-human antibodies may be labelled with a label. The label is any entity that is capable of being conjugated or bound to the anti-human antibody and that is capable of being detected by an analytical technique. The label may be conjugated or bound to the anti-human antibody prior to or after contacting the anti-human antibody with the antigen/sample.

[0094] Detection of the label is an indication that the human antibody is present in

the sample. If the label cannot be detected, then this is an indication that the human antibody is not in the sample. Since the presence, in the sample, of the human antibody to the antigenic SARS CoV protein is presumed to arise from SARS CoV infection, the presence or absence of the human antibody is an indication of whether the human is, or has been, exposed to the SARS CoV virus.

[0095] In one embodiment, the label may be a chemical moiety capable of being detected by an analytical technique, the chemical moiety being conjugated to the anti-human antibody. In this embodiment, the chemical moiety is generally conjugated to the anti-human antibody before the anti-human antibody is contacted with the antigen/sample.

[0096] In another embodiment the label may be another antibody or collection of other antibodies having conjugated thereto a chemical moiety that is capable of being detected by an analytical technique. In this embodiment, the other antibody or collection of other antibodies having the chemical moiety conjugated thereto is generally bound to the anti-human antibody after the anti-human antibody is contacted with the antigen/sample.

[0097] In a preferred embodiment, the method further comprises a washing step between steps (b) and (c), and more preferably between each of the steps of the method. Washing is preferably accomplished using a washing solution comprising a buffer, such as phosphate buffered saline solution containing about 1% normal serum from the animal species in which the antibody to which the chemical moiety is conjugated was prepared. An emulsifier may also be present in the washing solution.

[0098] A variety of chemical moieties capable of being detected by an analytical technique and capable of being conjugated to an antibody may be used in the label. For instance, the chemical moiety may be capable of fluorescence or radioactivity (see Fuller S.A., Evelegh M.J. & Hurrell J.G.R. (2000) Conjugates of Enzymes to Antibodies. In: Current Protocols in Molecular Biology. (Eds. Ausubel F.M., Brent R., Kingston R.E., Moore D.D., Seidman J.G., Smith J.A. & Struhl K.) John Wiley & Sons, Inc. Vol. 2, pp. 11.1.1-11.1.7, and, Sambrook J., Fritsch E.F. & Maniatis T. (1989) Molecular cloning. A laboratory manual. 2nd edn. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY, the disclosure of both being hereby

incorporated by reference). An example of chemical moiety useful in this invention is alkaline phosphatase. Further treatment may be required before the analytical technique is used to detect the label depending on the particular technique or chemical moiety being used.

[0099] Analytical techniques useful as detection methods are generally known in the art. For example, colorimetric, electrophoretic and radio-labelling techniques may be used (see Sambrook J., Fritsch E.F. & Maniatis T. (1989) Molecular cloning. A laboratory manual. 2nd edn. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY, the disclosure of which is hereby incorporated by reference).

[00100] Colorimetric techniques are generally preferred and may employ spectroscopic or visual verification of a colour change indicating a positive or negative test result. One skilled in the art will appreciate that other techniques may be employed as detection methods in the present invention.

[00101] The kit of the present invention may further comprise means for detecting the label, or such means may be separate from the kit.

[00102] By determining the presence of antibodies to S, M, E, N or U274, the presence or past presence of SARS CoV in a subject may be determined. Thus, the method of the present invention may be used to detect not only acute infection (one in which both the virus and antibody are present), but may also be used in cases wherein the antibody is present but there is no detectable virus, such as a subject that has recovered from SARS CoV infection.

[00103] The method and kit of the present invention may be used for field application such as a routine laboratory test for detection of SARS CoV infection, and where vaccination is not performed the test can detect asymptomatic SARS CoV carriers to obtain a realistic estimate of the prevalence of SARS CoV infection.

[00104] In one particular embodiment, the immunoassay is an ELISA. Antigenic protein GST-N (Δ111-118) and, optionally, antigenic protein GST-U274 (134-274) is immobilized onto the assay plate. Serum from a patient is contacted with the immobilised antigenic protein, and the assay plate is washed, blocked and washed in accordance with standard ELISA techniques described above. If the patient serum

is collected from a patient in early phase infection (between 2 and 11 days post-infection), anti-human IgA or anti-human IgM secondary antibody, both conjugated with horseradish peroxidase, is used to detect the presence of the antigen-primary antibody complex. If the patient serum is collected from a patient in late stage or convalescent stage infection (between 16 and 60 days post infection), anti-human IgG secondary antibody conjugated with horseradish peroxidase is used. A colour reaction is developed in the dark using the reagent tetramethylbenzidine, and is read in an ELISA plate reader at OD 450 nm.

[00105] The ELISA method of the present invention is both specific, yielding low numbers of false positive tests, and sensitive, yielding low numbers of false negative tests, particularly when using GST-N(Δ111-118) and when anti-human IgG secondary antibody is used. Furthermore, the minimum OD cutoff value for reading of the colour reaction may be adjusted. Thus, it maybe appropriate to set a higher cutoff value of 0.45 with a positive predictive value (PPV) of 100% for the ELISA when it is in use in low risk areas to minimize possibility of a false positive. Similarly, a lower cutoff value of 0.25 with an increased sensitivity and a higher negative predictive value (NPV) may be desired and appropriate when the ELISA is used in higher risk areas.

[00106] In another particular embodiment, the immunoassay is a rapid immunochromatography test. The antigenic proteins are independently immobilized in discrete lines on a nitrocellulose membrane, preferably GST-N (Δ111-118) and GST-U274 (134-274). A separate chromatography strip contains secondary antibody conjugated with gold colloidal particles at one end, with a separator in place to prevent early migration of the secondary antibody. The reagent-bearing pad is separated from a , and is separated from the nitrocellulose membrane. Upon addition of patient serum to the membrane, a releasing buffer is added to the chromatography strip, and the separator is removed to allow the secondary antibody to migrate into contact with the antigen-primary antibody complex, which has formed on the nitrocellulose membrane. A positive test result can be visualized typically in 2 to 15 minutes by the appearance of a colour line due to the formation of antigen-primary antibody-secondary antibody complex containing conjugated gold particles.

[00107] The rapid immunochromatography test requires little equipment to perform once the ready-made chromatography device is in hand. As with the above-described ELISA, the rapid immunochromatographic test is both sensitive and specific. In comparing results obtained with the two assays, an excellent agreement of 99.6% between the two was observed, with a kappa statistic of 1.00, and an excellent correlation, with an R^2 of 0.988 in relation to reactivity end. Thus, the rapid immunochromatographic test is a simple and rapid test that needs no special training to use, and provides diagnostic performance similar to that of an ELISA.

[00108] The same antigenic proteins used in the above-described ELISA may also be applied to the rapid immunochromatographic test, and may be applied separately as two different testing markers. Intrinsically, the newly developed rapid immunochromatographic test could provide not only indications for individual patient diagnosis, but because of the ability to test reactivity to individual antigenic proteins in a single test, this test may also provide population diagnosis details that may provide valuable epidemiological information for the spread of particular virus variants across a population, due to the greater variation of the U274 protein in comparison to the N protein.

[00109] In another particular embodiment, the immunoassay is a Western immunoblot. Generally, the antigenic proteins are transferred onto a membrane using standard techniques known in the art. As with the rapid immunochromatography test, individual antigenic proteins can be separated into discrete bands for use in the same test, allowing for detection of patient immune response to particular antigenic proteins at the same time. A strip of membrane containing antigenic proteins, preferably GST-N (Δ111-118) and GST-U274 (134-274). The probing of the membrane with patient serum and with secondary anti-human conjugated antibody are performed by standard Western blotting techniques. As above, the secondary antibody is chosen based on the time point during infection at which the patient sample is collected. The secondary antibody may be conjugated to the enzyme alkaline phosphatase, and the detection is then performed by developing a colour reaction using 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium.

[00110] The above-described Western immunoblot assay is more time

consuming and is not as easily adapted to high throughput of a large number of patient samples. However, it may be quite useful as a confirmatory test to confirm particular results obtained using another method of diagnosis.

[00111] In another aspect, the present invention also provides an antibody directed against the antigenic SARS CoV proteins. The antibody preferably is directed to regions of S, M, E, N or U274 that are unique to SARS CoV, to prevent cross-reactivity of the antibodies with antigens from other coronaviruses.

[00112] An antibody of the invention is either a polyclonal or monoclonal antibody having specificity toward an epitope contained in one of the antigenic SARS CoV proteins S, M, E, N or U274. Monospecific antibodies may be recombinant, *e.g.*, chimeric (*e.g.*, constituted by a variable region of murine origin associated with a human constant region), humanized (a human immunoglobulin constant backbone together with hypervariable region of animal, *e.g.*, murine, origin), and/or single chain. Both polyclonal and monospecific antibodies may also be in the form of immunoglobulin fragments, *e.g.*, F(ab)'2 or Fab fragments. The antibodies of the invention are of any isotype, *e.g.*, IgG or IgA, and polyclonal antibodies are of a single isotype or a mixture of isotypes.

[00113] Antibodies against the S, M, N, E or U274 proteins of SARS CoV, or homologs or fragments thereof, are generated by immunization of a mammal with a composition comprising said protein, homolog or fragment. Such antibodies may be polyclonal or monoclonal. Methods to produce polyclonal or monoclonal antibodies are well known in the art. For a review, see "Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, Eds. E. Harlow and D. Lane (1988), and D.E. Yelton *et al.*, 1981. Ann. Rev. Biochem. 50:657-680. For monoclonal antibodies, see Kohler & Milstein (1975) Nature 256:495-497.

[00114] The antibodies of the invention, which are raised to S, M, N, E or U274 proteins of SARS CoV, or homologs or fragments thereof, are produced and identified using standard immunological assays, *e.g.*, Western blot analysis, dot blot assay, or ELISA (see, *e.g.*, Coligan *et al.*, Current Protocols in Immunology (1994) John Wiley & Sons, Inc., New York, NY). The antibodies are used in diagnostic methods to detect the presence of antigenic SARS CoV protein S, M, E, N or U274 in

a sample, such as a biological sample. The antibodies are also used in affinity chromatography for purifying S, M, N, E or U274 proteins of SARS CoV, or homologs or fragments thereof.

[00115] Those skilled in the art will readily understand that an immune complex is formed between a component of the sample and the S, M, E, N or U274 protein or homologs or fragments thereof, or between the antibody of the invention and its target antigen, being S, M, E, N or U274 protein or homologs or fragments thereof, whichever is used, and that any unbound material is removed prior to detecting the immune complex. It is understood that the antigenic SARS CoV protein reagents are useful for detecting the presence of anti-S, M, E, N or U274 antibodies in a sample, *e.g.*, a blood sample, while an antibody of the invention is used for screening a sample, such as a gastric extract or biopsy, for the presence of SARS CoV polypeptides.

[00116] Briefly, for making monoclonal antibodies, somatic cells from a host animal immunized with antigen, with potential for producing antibody, are fused with myeloma cells, forming a hybridoma of two cells by conventional protocol. Somatic cells may be derived from the spleen, lymph node, and peripheral blood of transgenic mammals. Myeloma cells which may be used for the production of hybridomas include murine myeloma cell lines such as MPCII-45.6TGI.7, NSI-Ag4/1, SP2/0-Ag14, X63-Ag8.653, P3-NS-1-Ag-4-1, P.sub.3 X63Ag8U.sub.1, OF, and S194/5XX0.BU.1; rat cell lines including 210.RCY3.Agl.2.3; cell lines including U-226AR and GM1500GTGAl.2; and mouse-human heteromyeloma cell lines (Hammerling, et al. (editors), Monoclonal Antibodies and T-cell Hybridomas IN: J. L. Turk (editor) Research Monographs in Immunology, Vol. 3, Elsevier/North Holland Biomedical Press, New York (1981)).

[00117] Somatic cell-myeloma cell hybrids are plated in multiple wells with a selective medium, such as HAT medium. Selective media allow for the detection of antibodyproducing hybridomas over other undesirable fused-cell hybrids. Selective media also prevent growth of unfused myeloma cells which would otherwise continue to divide indefinitely, since myeloma cells lack genetic information necessary to generate enzymes for cell growth. B lymphocytes derived from somatic cells contain genetic information necessary for generating enzymes for cell growth but lack the

"immortal" qualities of myeloma cells, and thus, last for a short time in selective media. Therefore, only those somatic cells which have successfully fused with myeloma cells grow in the selective medium. The infused cells were killed off by the HAT or selective medium.

[00118] A screening method is used to examine for potential anti-S, M, E, N or U274 antibodies derived from hybridomas grown in the multiple wells. Multiple wells are used in order to prevent individual hybridomas from overgrowing others. Screening methods used to examine for potential anti-S, M, E, N or U274 antibodies include enzyme immunoassays, radioimmunoassays, plaque assays, cytotoxicity assays, dot immunobinding assays, fluorescence activated cell sorting (FACS), and other in vitro binding assays.

[00119] Hybridomas which test positive for anti-S, M, E, N or U274 antibody are maintained in culture and may be cloned in order to produce monoclonal antibodies specific for S, M, E, N or U274 . Alternatively, desired hybridomas can be injected into a histocompatible animal of the type used to provide the somatic and myeloma cells for the original fusion. The injected animal develops tumors secreting the specific monoclonal antibody produced by the hybridoma.

[00120] The monoclonal antibodies secreted by the selected hybridoma cells are suitably purified from cell culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-SEPHAROSE hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

[00121] The antibodies produce according to the present invention can be used to detect the presence of an antigenic SARS CoV protein or antigenic fragment thereof, including in diagnostic methods, identifying the presence of the particular antigenic protein in a patient sample or in a viral isolate obtained from a patient, as well as detecting purified recombinant antigenic SARS CoV proteins or antigenic fragments thereof. The antibodies may also be used in standard purification techniques, for example, immunoprecipitation. The antibodies provided by the invention may be used in immunoassays in accordance with known techniques, for example, capture ELISAs, Western immunoblots, or immunofluorescence assays, as

described above.

[00122] Thus, there is provided a method of detecting an antigenic SARS CoV protein, particularly S, M, E, N or U274, or an antigenic fragment thereof, using an antibody of the invention. A sample is contacted with a primary antibody directed against an antigenic SARS CoV protein, particularly S, M, E, N or U274 protein or an antigenic fragment thereof, for a time and under conditions sufficient for the antibody to form a complex with an antigen. If the antigenic protein is present in the sample, the primary antibody will bind to the antigenic protein, forming an antigen-primary antibody complex, which is then detected by the various methods described above, for example by using a secondary antibody directed against the primary antibody, which may be conjugated to a detection molecule.

[00123] All documents referred to herein are fully incorporated by reference.

EXAMPLES

[00124] **Example 1: Use of SARS CoV proteins as diagnostic markers**

[00125] ***Materials And Methods***

[00126] ***Materials:*** All reagents used in this study were purchased from Sigma (St. Louis, Mo.), unless otherwise stated. All cell lines were purchased from the American Type Culture Collection (Manassas, Va.) and were cultured at 37°C in 5% CO_2 in Dulbecco's modified Eagle medium containing 1 g of glucose/liter, 2 mM L-glutamine, 1.5 g of sodium bicarbonate/liter, 0.1 mM non-essential amino acids, 0.1 mg of streptomycin/ml, 100 U of penicillin, and 5% fetal bovine serum (HyClone, South Logan, Utah).

[00127] ***Construction of plasmids:*** For transient expression in mammalian cells, the vectors used were pXJ40HA, for tagging proteins at the N terminus with one hemagglutinin (HA) epitope (15), and pXJ40-3_HA, for tagging proteins with three HA epitopes at the C terminus. For the expression of glutathione S-transferase (GST) fusion proteins in bacteria, genes were cloned in frame with GST in pGEX-4T-1 (Amersham Pharmacia Biotech, Uppsala, Sweden). For the stable transfection of S in CHO cells, a full-length S construct tagged at the C-terminal end with green

fluorescent protein (GFP) was cloned into the pMMTC vector as described in GB 2314332. All of the constructs used for this study are listed in Table 1.

Table 1 Plasmids used for this study

Plasmid	Nucleotide positions in ORF	Amino acid positions	Total no. of amino acids
pXJ40HA-U274	400–822	134–274	141
pXJ40-E-3'HA	1–228	1–76	76
pXJ40-M-3'HA	1–663	1–221	221
pXJ40-U122-3'HA	1–366	1–122	122
pXJ40HA-N	203–1266	69–422	354
pMMTC-S-GFP	1–3765	1–1255	1,255
pGEX-4T1-U274	400–822	134–274	141
pGEX-4T1-U122	46–333	16–111	96
pGEX-4T1-N	357–1266	120–422	303

[00128] SARS-CoV 2003VA2774, an isolate from a SARS patient in Singapore, was used for this study. For the cloning of various genes, RNAs were extracted by use of a Qiagen viral RNA kit (Valencia, Calif.) from a SARS-CoV-infected Vero E6 cell culture supernatant harvested when the cultures showed at least 75% cytopathic effects. Reverse transcription was performed with Superscript II™ Rnase H⁻ reverse transcriptase (Gibco BRL, Gaithersburg, Md.) and an oligo(dT) primer. PCRs were performed with either HotStar™ polymerase (Qiagen), Titanium™ *Taq* DNA polymerase (Clontech Laboratories Inc., Palo Alto, Calif.), or High Fidelity™ *Taq* polymerase (Roche Molecular Biochemicals, Indianapolis, Ind.). In some cases, overlapping cDNAs provided by the Genome Institute of Singapore (isolate SIN2774; accession no. AY283798) were used as templates instead. All of the primers used for this study were synthesized by Genset Singapore Biotech (Singapore). The sequences of all constructs used in this study were confirmed by DNA sequencing performed at the core facility at the Institute of Molecular and Cell Biology by the dideoxy method with a *Taq* DyeDeoxy™ terminator cycle sequencing kit and an automated DNA sequencer (model 373) from PE Applied Biosystems (Foster City, Calif.).

[00129] ***Transient transfection of mammalian cells:*** Transient transfection experi-ments were performed with Effectene™ transfection reagent (Qiagen)

according to the manufacturer's protocol. Typically, ~10^6 COS-7 cells were plated on a 6 cm diameter dish and allowed to attach for at least 4 h. 1 to 2 μg of DNA was used per plate, and the cells were left for at least 14 h before the cells were washed with phosphate-buffered saline (PBS), lysed directly in Laemmli's sodium dodecyl sulfate (SDS) buffer, and used for Western blot analysis.

[00130] ***Expression of GST fusion proteins:*** Exponentially growing cultures (optical density at 600 nm of ~0.7) of *Escherichia coli* (BL21/DE3) cells harboring the pGEX-4T-1 expression constructs were induced to synthesize fusion proteins by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), after which the cells were allowed to grow for another 4 h at 37°C or 12 h at 30°C. Cells were harvested, resuspended in PBS containing 0.5% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride, and then sonicated with an ultrasonic processor (Misonix Inc., Farmingdale, N.Y.). GST fusion proteins were then purified from the lysate by use of glutathione (GSH)-Sepharose beads (Amersham Pharmacia Biotech). Proteins were eluted from the beads with 10 mM reduced GSH in 50 mM Tris-HCl, pH 9.2, and 0.1% SDS, and protein concentrations were determined by use of a Coomassie Plus™ assay kit (Pierce, Rockford, Ill.). Proteins were also separated in SDS-polyacrylamide gels and stained with 0.25% Coomassie brilliant blue R-250 (Bio-Rad, Hercules, Calif.) in 45% methanol and 10% acetic acid.

[00131] ***Western blot analysis:*** For Western blot analysis, approximately 10^5 transfected COS-7 cells or 50 ng of GST fusion proteins was separated in SDS-polyacrylamide gels and transferred to nitrocellulose Hybond C membranes (Amersham Pharmacia Biotech). The membranes were blocked with 5% nonfat dry milk. For the detection of HA-tagged or GST fusion proteins, the membranes were incubated with either an anti-HA polyclonal or anti-GST monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.) overnight at 4°C and washed extensively with PBST (PBS containing 0.05% Tween 20), followed by incubation with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Pierce) for 1 h at room temperature. Membranes were then washed extensively with PBST, and the detection of signals by an enhanced chemiluminescence method (Pierce) was performed. For patient sera, each sample was first treated with 0.5% Triton X-100 and 0.1 mg of RNase (Sigma)/ml and then diluted 1:150 to 1:500 with PBST containing

1% nonfat dry milk. After incubation for 1 to 3 h at room temperature or overnight at 4°C, the membranes were incubated with an anti-human HRP-conjugated immunoglobulin G (IgG) (Santa Cruz Biochemicals), IgA, or IgM (Zymed Laboratories Inc., San Fran-cisco, Calif.) antibody for 1 h at room temperature, followed by detection as described above. All secondary antibodies were used at a 1:2000 dilution. Due to the limited amount of patient sera from the seven pairs of sera, Western blots of a mixture of three GST fusion proteins, N, U274, and U122, and GST were run in a single-slot SDS-10% polyacrylamide gel electrophoresis (PAGE) gel and transferred onto nitrocellulose membranes. The membranes were cut into strips of about 0.8 mm wide, and each strip was probed with 300 to 400 μl of diluted sera. For the late time point sera, the strips were incubated with the diluted sera for 1.5 h at room temperature. For the early time point sera, the strips had to be incubated overnight with the sera, at room temperature, to detect any signal. The secondary antibodies were incubated for 1 h at room temperature. This method was also used when large numbers of samples were being screened.

[00132] ***Expression of S-GFP fusion protein in CHO cells and immunofluorescence analysis to determine anti-S immunoreactivity in patient sera:*** CHO cells were transfected with pMMTC-S-GFP by use of DMRIE-C reagent (Gibco BRL) according to the manufacturer's protocol. Transfected cells were selected in Geneticin™ (Gibco BRL) for ~1 week. Cells were then analyzed under a Zeiss microscope (Carl Zeiss Vision GmbH, Hallbergmoss, Germany), and the clone with the strongest expression signal was picked and grown in medium containing 100 μM $ZnSO_4$. Zn^{2+} ions increase the expression of genes by the pMMTC vector (see GB 2314332) . Cells were dislodged from the plates with 0.04% EDTA, seeded onto black Teflon Menzel diagnostic slides (Merck), and blown dry, followed by fixing in acetone at ~20°C for 1 h. Then the cells were incubated with sera at dilutions of 1:20, 1:40, 1:80, and 1:160 (in PBS) for 1.5 h at 37°C, followed by incubation with a fluorescein isothiocyanate (FITC)- or rhodamine (Rh)-conjugated anti-human IgG (Sigma) at a 1:20 dilution for 1.5 h at 37°C. When Rh-conjugated anti-human IgG was used, it was diluted in PBS, and when FITC-conjugated anti-human IgG, IgM (Sigma), or IgA (Dako A/S, Glostrup, Denmark) was used, it was diluted in 0.05% Evans Blue solution (Fluka, Buchs, Switerland), which blocks GFP fluorescence from transfected cells. Slides were then viewed under a Zeiss microscope (Carl Zeiss

Vision GmbH) and scored as follows: +++, very strong staining; ++, moderate staining; and +, weak staining.

[00133] ***Collection of sera from SARS-CoV-infected patients:*** The first six serum samples (from patients 1 to 6), except for that from patient 3, were obtained by plasmapheresis. Plasmapheresis was performed on patients who (i) had previous documented SARS-CoV infection according to World Health Organization (WHO) criteria, (ii) had been in the convalescent phase for at least 6 weeks, and (iii) were symptom-free and willing to participate in the study. Blood was taken from patient 3, and the serum was separated out for the experiments. All participants have given written consent, and approval from the Tan Tock Seng Hospital Ethics Committee has been granted. The second set of sera consisted of seven pairs of samples that were collected from patients admitted to Tan Tock Seng Hospital or Singapore General Hos-pital. These patients were admitted upon the onset of illness, as defined by WHO criteria, and the dates of admission and dates of collection of blood are shown in Table 2. The first samples (set A) were collected 2 to 11 days after the onset of illness, and the second set of samples (set B) were collected 16 to 54 days after the onset of illness.

Table 2 Description of sera collected from seven SARS CoV infected patients

Patient	Sample	Date of onset of illness	Date of sample collection	No. of days after onset of illness
D2	2A	18 March 2003	20 March 2003	2
	2B	18 March 2003	15 April 2003	28
D3	3A	17 March 2003	20 March 2003	3
	3B	17 March 2003	17 April 2003	31
D4	4A	16 March 2003	20 March 2003	4
	4B	16 March 2003	31 May 2003	48
D5	5A	2 April 2003	7 April 2003	5
	5B	2 April 2003	29 April 2003	27
D8	8A	19 March 2003	27 March 2003	8
	8B	19 March 2003	4 April 2003	16
D9	9A	9 March 2003	18 March 2003	9
	9B	9 March 2003	2 May 2003	54
D11	11A	24 February 2003	7 March 2003	11
	11B	24 February 2003	3 April 2003	38

[00134] The last set of sera was obtained from 61 probable SARS patients who were discharged from Tan Tock Seng Hospital. For some of these patients, sera were taken upon discharge (3 to 4 weeks after the onset of illness), while most of the patients were recruited >3 weeks after discharge (>6 weeks after the onset of illness)

(see Table 3 for details). For one patient (patient P3L), a serum sample was taken 111 days (3 1/2 months) after the onset of illness. Samples from two other patients (P7 and P8) were obtained by plasmapheresis. A control serum was purchased from Sigma, and 99 serum samples were obtained from healthy donors who have given informed consent. These healthy donors (i) did not have a diagnosis of SARS, suspect SARS, or have contact with a person who was served a home quarantine order for SARS; (ii) did not have a fever, symptoms of influenza, runny nose, or sore throat within the last week at the time of donation; (iii) had not been on immunosuppressants; (iv) had no significant medical illnesses; and (v) had no history of travel to SARS-affected areas since November 2002.

[00135] ***Results***

[00136] ***Detection of IgG antibodies against viral proteins encoded by the SARS-CoV genome in the sera of probable SARS patients:*** In order to assess which of the viral proteins encoded by the SARS-CoV genome may be exploited as diagnostic anti-gens for the development of a serological assay to detect SARS-CoV infection, we cloned three of the four structural proteins (E, M, and N) into expression vectors. Due to the large size (~4.7 kbp) and heavy glycosylation of the spike (S) protein, it was analyzed separately by an immunofluorescence method as described below. In addition, two of the SARS-CoV unique proteins (U274 and U122) were also included in this study, and they will be referred to hereafter as UX, where "X" stands for the number of predicted amino acids of the proteins. In this study, we used only the C-terminal hydrophilic region of U274, as it has three potential transmembrane domains at its N-terminal end and these hydrophobic regions may affect the solubility of a recombinant U274 protein. The positions of the structural and unique proteins and their corresponding ORF numbers, as designated by Marra and coworkers (5), are shown in Figure 1.

[00137] This panel of HA-tagged proteins was expressed in COS-7 cells by transient transfection, and total protein lysates were analyzed by Western blot analysis with sera from three convalescent patients (Figure 2, patients 1 to 3) to determine if these sera had any antibodies against the expressed proteins. Plasmapheresis was performed on patients 1 and 2, and the plasmapheresis products were used in Western blot analysis, while for patient 3, serum was used for the analysis.

[00138] All three patients had antibodies against the N protein (aa 69 to 422), but not against the other structural proteins, M and E. Interestingly, the C-terminal hydrophilic region of U274 (aa 134 to 274) was also detected by the sera of patients 1 and 3 but not by the serum of patient 2 (Figure 2). A control serum did not detect N, U274, or any other proteins. U122 was also not detected by any of the three sera, suggesting that it may not be expressed, it is not a structural protein, or it is not sufficiently antigenic.

[00139] As bacterially expressed proteins are easier and cheaper to produce on a large scale, we next expressed N and U274 as GST fusion proteins and tested them for reactivity to patient sera. Coomassie staining showed that GST-N (aa 120 to 422) and GST-U274 (aa 134 to 274) were of > 90% purity after a one-step purification with GSH-Sepharose beads (Figure 3). The GST-N (aa 120 to 422) protein used here lacked the N-terminal part of the N protein, which contains a highly conserved motif (FYYLGTGP; aa 111 to 118 of SARS-CoV N) found in all coronaviruses (10), so there would be less chance of cross-reactivity with antibodies against other coronaviruses. From a 400-ml bacterial culture, ~10 mg of GST-N and ~2 mg of GST-U274 could be obtained, respectively. GST-U122 (aa 16 to 111, lacking the signal peptide at the N terminus and the hydrophobic C terminus) and GST were also expressed and used as controls. The GST fusion proteins were probed with the same three patient sera and one control se-rum, as for the mammalian expressed proteins (Figure 2). Consistent with the proteins expressed in COS-7 cells, GST-N was clearly detected by all three patient sera and GST-U274 was detected only by the sera from patients 1 and 3 (Figure 4). Neither GST-U122 nor GST showed any background with all three patient sera, and there was no nonspecific binding of the control serum to any of the proteins (Figure 4). Sera from another three convalescent patients (patients 4 to 6) were also tested, and they were reactive specifically towards both GST-N and GST-U274, but not to GST-U122 or GST (Figure 4).

[00140] Profile of IgG, IgM, and IgA antibodies against N and U274 proteins in seven pairs of sera obtained at two time points of infection: A set of paired sera from seven patients were also examined for reactivity against GST-N and GST-U274. These were obtained at two time points of infection, one at 2 to 11 days after the onset of illness (set A) and another at 16 to 54 days after the onset of illness (set B) (Table

2). For all seven cases, the second time point samples were taken at least 8 days after the first time point samples. As shown in Figure 5, anti-IgG antibodies against GST-N protein were present in all seven patients' sera at the later time point (set B), but not at the early time points (set A). For both sets of sera, the membranes were incubated with the diluted sera (1:150) for 1.5 h at room temperature, followed by incubation with the secondary antibody (HRP-conjugated anti-human IgG) for 1 h at room temperature. In addition, all seven of the late time point sera also contained antibodies against GST-U274, albeit at a lower level than that for GST-N (Figure 5, set B).

[00141] We repeated the experiment for the early time point sera (set A) but incubated the blots with diluted sera overnight at room temperature instead of for 1.5 h and used three different secondary antibodies, anti-IgG, anti-IgM, and anti-IgA, and the results for each of them are shown in Figure 6. For IgG, only samples 9A and 11A showed some reactivity to GST-N. For IgA, though, all seven samples showed reactivity to GST-N, and the signals were particularly strong for samples 9A and 11A. For IgM, all seven samples showed a low level of reactivity to GST-N. As the early time point sample for each patient was collected a different number of days after the onset of illness, our results suggest that by as early as 2 days after the onset of illness (patient D2, sample 2A), IgM and IgA anti-bodies against the N protein were present. By 9 days after the onset of illness, very high levels of IgA antibodies against N were present and IgM and IgG antibodies were also detectable (Figure 6, samples 9A and 11A). As for GST-U274, some reactivities were observed for IgA (samples 5A, 8A, 9A, and 11A) and for IgM (sample 11A) from 5 to 11 days after the onset of illness. Interestingly, quite a strong signal for GST-U274 was observed for sample 11A (11 days after the onset of illness) when IgM was used, and for the corresponding early sample, 11B, the signal for GST-U274 was also strong (Figures 5 and 6).

[00142] ***Determination of the sensitivity and specificity of immunoreactivity (IgG) against N and U274 proteins:*** In order to determine the sensitivity and specificity of the immunoreactivity against N and U274, we obtained another 61 samples from probable SARS patients who were discharged from hospitals and tested them for the presence of IgG antibodies against N and U274 by Western blot analysis. As shown in Table 3, the samples were taken from 31 to 111 days after the onset of illness. (Legend for Table 3: [a] Determined by Western blot analysis; +++, ++, +,

very strong, moderate, and weak signals, respectively, were observed on the autoradiograph; — , no signal was observed; [b] Determined by an immunofluorescence method in which CHO cells stably expressing the S protein were incubated with a diluted patient serum (1:40) followed by a FITC-conjugated anti-human IgG antibody—slides were then viewed under a Zeiss microscope and scored as follows: +++, very strong staining; ++, moderate staining; +, weak staining. One hundred sera from healthy volunteers were examined at the same dilution, and none of them showed any staining; [c] Samples were obtained at the stated number of days after the onset of illness; [d] Samples from two healthy contacts (patients 868 and 873) were also examined; [e] Samples obtained by plasmapheresis; [f] Late time point from one of the seven pairs of sera described in Table 2.)

Table 3 Reactivities of serum samples collected from 74 probable SARS patients

Patient no.	Reactivity to N protein[a]	Reactivity to U274 protein[a]	Reactivity to S protein[b]	No. of days after onset of illness[c]
174	+++	++	+	49
318	+++	—	+	53
350	+++	++	+	62
358	+++	+	+	60
377	+++	+	+	61
387	+++	—	+	63
432	+++	++	+++	42
442	+++	+	++	63
487	+++	+	++	54
492	+++	+	+	31
526	+++	+	+	63
541	+++	+	++	58
546	+++	++	++	62
561	+++	++	+	57
566	+++	+	++	59
571	+++	+	+	57
576	+++	+	++	63
581	+++	—	++	53
586	+++	++	++	38
596	+++	—	+	39
603	+++	++	++	67
621	+++	++	+	50
633	+++	—	+++	39
638	+++	+	+	60
644	+++	+	++	41
672	+++	+	+	53
677	+++	++	++	59
682	+++	—	+	58
687	+++	+	++	61
696	+++	—	+	41
701	+++	++	++	40
706	+++	+	++	39
711	+++	—	+	54
716	+++	—	++	40
726	+++	+	++	34
734	+++	—	+	41
739	+++	—	++	49
744	+++	+	++	68

Table 3 (con't)

Patient no.	Reactivity to N protein[a]	Reactivity to U274 protein[a]	Reactivity to S protein[b]	No. of days after onset of illness[c]
749	+++	+++	++	59
759	+++	+	+	42
764	+++	+	++	43
769	+++	+	++	64
774	+++	+	++	62
784	+++	–	+++	38
789	+++	–	++	55
793	+++	+	+	48
798	+++	–	++	44
803	+++	–	++	40
823	+++	+	++	37
840	+++	++	+++	43
845	+++	++	++	36
859	+++	+++	++	55
868[d]	–	–	–	Not applicable
873[d]	–	–	–	Not applicable
878	+++	++	+++	58
883	+++	+	++	56
888	+++	+	++	33
893	+++	++	+++	41
4153	+++	–	++	41
4209	+++	–	++	21
P3L	+++	++	+	111
P1[e]	+++	++	++	>42
P2[e]	+++	–	+	>42
P3	+++	++	+	>42
P4[e]	+++	++	+	>42
P5[e]	+++	++	+	>42
P6[e]	+++	++	+	>42
P7[e]	+++	+	+	>42
P8[e]	+++	++	++	>42
2B[f]	+++	+	++	28
3B[f]	+++	++	+++	31
4B[f]	+++	+	+	48
5B[f]	+++	+	+++	27
8B[f]	+++	+	+++	16
9B[f]	+++	+	++	54
11B[f]	+++	++	++	38

[00143] Plasmapheresis products from 2 of the patients (P7 and P8) and sera from the other 59 patients were used. All of the samples showed strong immunoreactivity against the N protein (100%), and 44 of the 61 samples (72%) were positive for U274 (Table 3). In general, the signals observed for the U274 protein were much lower than those for the N protein. The exceptions were samples from patients 749 and 859, for which the signals for the U274 and N proteins were equally strong. Another 99 samples from healthy donors were also tested, and none of them showed any immunoreactivity to N or U274. In addition, samples from two patients (patients 868 and 873) who had close contact with probable SARS patients but never developed any of the clinical symptoms also did not show any immunoreactivity to N or U274 (Table 3). Therefore, the immunoreactivity against the N protein is highly

sensitive (found in 100% of a total of 81 samples from probable SARS patients) and specific.

[00144] ***Detection of IgG antibodies against SARS-CoV S protein in convalescent-phase sera by immunofluorescence:*** Due to the large size and heavy glycosylation of the S protein, it is advantageous to express this protein in mammalian cells instead of bacteria, as it is then possible to detect antibodies that may be dependent on conformation and/or glycosylation of the S protein. CHO cells were stably transfected with a full-length S construct tagged at the C-terminal end with GFP. After selection with antibiotics, a pool of clones expressing significant levels of S protein, as indicated by GFP fluorescence, were obtained (Figure 7). These CHO cells were used for an immu-nofluorescence staining method to determine if there were IgG antibodies against the SARS-CoV S protein in convalescent-phase sera. Briefly, the cells were fixed with acetone and then incubated with diluted patient sera, followed by incubation with an Rh-conjugated anti-human IgG antibody.

[00145] As shown in Figure 7, sera from patients 1 to 6 all contained IgG antibodies against S, as detected by immunofluorescence, and no signal was observed when a control serum was used. All remaining samples were tested similarly, except that the Rh-conjugated anti-human IgG antibody was replaced with a FITC-conjugated one in the presence of a high concentration of Evans Blue solution (0.05%). This is because of the greater ease of judging cells stained with FITC than those stained with Rh, and this concentration of Evans Blue solution was sufficient to block out all of the GFP fluorescence in the trans-fected cells (data not shown).

[00146] As shown in Table 3, 100% of the convalescent-phase sera (74 samples) showed immunoreactivity against S at a dilution of 1:40, and 100 samples from healthy donors did not show any signal at the same dilution. The lowest dilution tested was 1:20, but a few of the samples from healthy donors showed weak signals at this concentration; therefore, the results from the 1:40 dilution were used for comparison. At higher dilutions (1:80 and 1:160), weaker signals were observed for most of the patient sera, as would be expected. In particular, for samples 432, 633, 784, 840, 878, 893, 3B, 5B, and 8B, for which very strong signals (+++) were observed at a 1:40 dilution, the signals decreased gradually at higher dilutions, i.e., moderate signals (++) were observed at 1:80 and weak signals (+) were observed at

1:160. The seven early time point samples (Table 2, set A) were also analyzed in the same manner, but with IgG, IgM, and IgA secondary antibodies separately, but none of them showed any reactivity (at a 1:40 dilution) (data not shown).

[00147] **Example 2: ELISA and Immunochromatographic Assays**

[00148] ***Materials and Methods***

[00149] ***Recombinant proteins:*** The materials and methods used for obtaining the recombinant proteins are as described for Example 1 above. For this study, the Gst-U274 protein was further purified with a Superdex™ S-200 HR10/30 column on an AKTA fast protein liquid chromatography (FPLC) system (Amersham). The buffer used contained 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 6 M urea, and 1 mM β-mercaptoethanol; the flow rate was 0.5 ml/min; and fractions of 1 ml were collected. Fractions 12 and 13 were combined and dialyzed against phosphate-buffered saline (PBS) overnight with at least three changes of buffer.

[00150] ***Serum specimens:*** Seventy-four convalescent-phase serum samples were collected from SARS patients admitted to the Tan Tock Seng Hospital or the Singapore General Hospital. Ninety-one control serum samples were obtained from healthy local donors who worked at the Institute of Molecular and Cell Biology, Singapore, Republic of Singapore. All specimens were collected with consent, and patient samples were collected at 16 to 65 days from the onset of symptoms. In addition, 119 sera from healthy donors purchased from BioClinical Partners, Inc. (Franklin, Mass.), were included in the study as additional healthy controls.

[00151] ***ELISA:*** The Gst-N and GstT-U274 proteins were pre-diluted in carbonate buffer (pH 9.6) at final concentrations of 0.1 and 0.15 μg/ml, respectively, prior to plate coating. The plates were prepared as described (16). Briefly, the 96-well polystyrene microtiter plates (Immuno 1B; Themo Labsystem, Franklin, Mass.) were coated with the protein mixtures at a volume of 100 μl per well by incubation overnight (16 to 18 h) at room temperature. The plates were washed five times with PBS-Tween 80 (PBST), and nonspecific binding sites were blocked with 200 μl (per well) of a Tris-based diluent for 1 h at room temperature. The plates were further washed another five times before 10 μl of serum in 200 μl of Tris-based diluent

(containing 1% each bovine serum albumin [BSA] and skim milk powder) was added. Subsequently, the plates were incubated for 30 min at 37°C, followed by six washes with PBST. Horseradish peroxidase-conjugated goat anti-human immunoglobulin G (IgG; 1:500 dilution) was added at 100 µl per well, and this mixture was incubated for 30 min at 37°C. The plates were then washed six times in PBST and color development proceeded with the addition of the enzyme substrate tetramethylbenzidine (TMB) at 100 µl per well. After a 15-min incubation in the dark at 37°C, the reaction was stopped by adding 100 µl of 1 N HCl per well. The optical densities (OD) were measured at 450 nm with a 620-nm reference filter.

[00152] ***Rapid immunochromatographic test:*** The membrane-based immunochromatographic test device consisted of a chromatography strip, a separator, and an absorbent pad, all housed in a cassette as described previously in US Patent No. 6,316,205, and the chromatography strip was prepared separately according to the procedure detailed in that reference with slight modification before assembly into the device. Briefly, a nitrocellulose membrane with an average pore size of 8 µm (Whatman, Ann Arbor, Mich.) was sprayed with the Gst-N and the Gst-U274 recombinant antigens in two separate lines, at concentrations of 0.1 and 0.15 mg/ml, respectively, with a BioDot (Irvine, Calif.) spraying machine. The membrane was dried for 10 min before being immersed for 1 min in a blocking buffer consisting of Milli-Q purified water with 6.7% StabilCoat™ (SurModics, Inc.), 0.05% Triton X-100, and 0.5% casein. The blocked membrane was then dried at 37°C for 60 min before being affixed to a membrane backing. The reagent-bearing pad was prepared using a porous matrix. The porous matrix was sprayed with goat anti-human IgG antibodies that were labeled with colloidal gold particles 25 to 30 nm in diameter. This reagent-bearing pad was then dried at 37°C for 2 h prior to incorporation into the device. A chromatographic card was prepared by affixing a 0.1% Triton X-100-treated porous matrix to one end of the nitrocellulose strip and the reagent-bearing pad to the other end on the same membrane backing. This assembly was then cut into a strip approximately 4 by 56 mm^2 in size. An assay device was assembled by placing an absorbent pad at the bottom half of the cassette and then a separator, followed by one unit of the chromatographic strip before closing the top half of the cassette. In addition, a reagent-releasing wash buffer was also prepared with Milli-Q purified

water with 50 mM NaH2PO4, 300 mM NaCl, and 0.1% sodium dodecyl sulfate (pH 8.0).

[00153] When testing, 25 μl of undiluted serum sample was added to the specimen window of the assay device. The sample was allowed to migrate laterally and cover part of the membrane. When the sample reached the indicator in the viewing window in approximately 30 s, three drops of reagent-releasing washing buffer were added to the buffer window to release the colloidal gold-labeled goat anti-human IgG antibodies. The separator was then removed by pulling the protruding end to allow the chromatographic element and the absorbent pad to come into contact. The colloidal gold-labeled goat anti-human IgG antibodies were then allowed to migrate across the chromatographic strip completely. The result can be read in typically 2 to 15 min through the viewing window. A negative result will be indicated by the appearance of a control line only, whereas with a positive result, the control line and either or both of the test lines appear in the viewing window (Figure 8).

[00154] ***Statistical analysis:*** The kappa statistic was used to measure the strength of agreement between the results by the new rapid test and the new ELISA. Kappa statistic values of > 0.75, 0.40 to 0.75, and < 0.40 represent excellent agreement, good to fair agreement, and poor agreement, respectively (17). For the data analysis for ELISA, an arbitrary cutoff value of 0.45 was selected but confirmed with the delta values (7), which could provide an indication of an optimal differentiation between the positive and negative populations.

[00155] ***Results***

[00156] ***ELISA:*** When tested at a sample dilution of 1:20, the ELISA detected IgG antibodies to SARS-CoV in 100% (n = 74) of the convalescent-phase samples from the SARS patients, with a mean ± standard deviation OD value of 2.32 ± 0.54 (Table 4 and Figure 9). In contrast, only 1 initial-positive sample was found by the test out of the 210 control sera from the healthy donors with a mean ± standard deviation OD value of 0.05 ± 0.05 ($P < 0.001$) (Figure 9). The test thus provided a PPV of 98.7% and a negative predictive value (NPV) of 100% (Table 4). In addition, the ELISA gave a positive delta of 5.4 and a negative delta of 3.6, indicating excellent differentiation between positives and negatives. Furthermore, and as shown in Figure

10, when titration curves were obtained with seven samples from the SARS patients by using this new ELISA, the reactivity end point was found at dilutions ranging from > 1:40 to 1:640 with different samples.

Table 4 Performance of individual markers

Test format	SARS patients		Healthy controls		PPV (%)	NPV (%)
	No. positive/ total	% Sensitivity	No. positive/ total	% Specificity		
ELISA (combined)[a]	74/74	100	1/210	99.5	98.7	100
Rapid test						
Gst-N	42/42	100	2/210	99.0	95.3	100
Gst-U274	36/42	85.7	0/210	100	100	97.2
Combined	42/42	100	2/210	99.0	95.3	100

[a] Gst-N plus Gst-U274.

[00157] Rapid immunochromatographic test. When tested with undiluted samples, the Gst-N and the Gst-U274 proteins used in the rapid test reacted to IgG antibodies in 100% (42 of 42) and 85.7% (36 of 42), respectively, of the sera from SARS patients who met the WHO criteria for SARS (Table 4). Thus, the overall detection rate for the new test was 100% (42 of 42) (Table 4). Only the Gst-N protein in the rapid test was found to cross-react with 2 of the 210 sera from the healthy controls. The test was therefore shown to have specificity, PPV, and NPV of 99.0, 95.3, and 100%, respectively, with the tested populations (Table 4). When the results were compared, the rapid test and the ELISA gave an excellent agreement of 99.6%, with a kappa statistic of 1.00 (Table 5). In addition, an excellent correlation ($R2$ = 0.988) was found when the reactivity end points of the rapid test (at dilutions ranging from 1:8 to 1:128) and the ELISA were compared by using the same seven patient samples (Figure 11).

Table 5 Agreement between the immunochromatographic test and the ELISA test

Rapid test	No. of ELISA results		% Agreement	Kappa statistic[a]
	Positive	Negative		
Positive	43	1	99.6	1.00
Negative	0	208	100	

[a] A kappa statistic of ≥0.75 represents excellent agreement, 0.40 to 0.75 represents good-to-fair agreement, and <0.40 represents poor agreement (11).

[00158] **Example 3: Evaluation of ELISA and Immunochromatography Assays**

[00159] *Materials and Methods*

[00160] ***Serum specimens:*** Serum specimens were collected from patients who presented with clinical suspected SARS according to the WHO definition (18) and who were admitted to three acute regional hospitals in Hong Kong between 18 March and 24 May 2003. A total of 227 serum samples from these patients was tested using an IF test (9) and confirmed to have IF titers of >1:10 to 1:2560 dilution. In the meantime, 385 serum samples from healthy donors collected locally in Hong Kong were used as controls. In addition, 1066 sera from healthy donors purchased from BioClinical Partner Inc. (Franklin, MA) were included in the study as additional healthy controls. For the disease controls, Genelabs Diagnostics (GLD, Singapore) archive serum samples from various previous studies were used and these included 50 samples each from patients who had non-SARS related fever (confirmed as dengue fever) or suffered non-SARS related respiratory illness (confirmed as tuberculosis).

[00161] ***Immunofluorescent test:*** The IF test was prepared and carried out as described above in Example 1. Briefly, smears of SARS CoV-infected Vero cells were prepared, fixed in acetone for 10 min, and stored at –80°C before use. Batches of smears with 60 to 70% SARS CoV infected cells were confirmed with a high titer positive control serum samples before use. Patient samples prepared in serial twofold dilutions starting with 1:10 were added to the smears and incubated for 30 min at 37°C. The smears were washed two times in PBS before a further incubation for 30 min at 37°C with a goat anti-human IgG labeled with fluorescein isothiocyanate. A sample was scored as positive if the fluorescent intensity was equal to or higher than that of a weakly positive control included in the study.

[00162] ***ELISA:*** The ELISA was produced by Genelabs Diagnostics Pte Ltd in Singapore utilizing two recombinant proteins (Gst-N and Gst-U274) as previously described for Example 2 above. The assays were carried out following strictly the instruction provided. Briefly, the ELISA plates were added with 10µl of serum in 200µl/well of Tris-based diluent containing BSA and skim milk powder and incubated for 30 min at 37°C followed by six washes with PBST. An HRP-conjugated

goat anti-human IgG (1:500 dilution) was added at 100µl per well and incubated for a further 30 min at 37 °C. The plates were then washed six times in PBST and allowed a color development with the addition of 100µl per well of enzyme substrate TMB (tetramethylbenzidine). After a 15-min incubation in the dark at 37 °C, the reaction was stopped by adding 100µl per well of 1N HCl. The optical densities (OD) were measured at 450nm with a 620nm reference filter.

[00163] ***Rapid Immunochromatographic Test:*** Again, the membrane-based test device was produced at Genelabs Diagnostics Pte Ltd in Singapore following the procedure previously described for Example 2. The device consisted of a chromatography strip, a separator and an absorbent pad all housed in a cassette as described.

[00164] The chromatography strip was deposited with two SARS-specific recombinant proteins: Gst-N and Gst-U274 separately following a detailed disclosure of Guan et al (19, 20). Test samples if contained antibody to SARS-CoV would bound to the either or both of the immobilized recombinants and the immunocomplex formed can be detected by the immobilized colloidal gold labelled with anti-human IgG when the latter was released by a reagent-releasing and washing buffer (19).

[00165] The assays were also carried out following strictly the instruction provided by the manufacturer (19). Briefly, a 25µl of serum sample was added to the sample well and allowed to migrate laterally to cover a portion of the membrane in the result-viewing window. Three drops of the reagent-releasing and washing buffer were added to the second well when the serum sample-wetting front reached the blue indicator line in the viewing window. The separator tab was pulled until resistance was felt and an additional drop of the reagent-releasing and washing buffer was then added to the sample well.

[00166] The results can be read in typically 2-15 min through the viewing window but were all recorded at 15 min. A sample was scored as negative when only the control line appeared but positive when the control line and either or both of the test lines were seen in the viewing window (19).

[00167] ***Statistical analysis:*** Delta values which are defined as the distance of

the mean OD ratio of the sample population from the cutoff measured in standard deviation units (7) were calculated for validating the cutoff values. The kappa statistic was used to measure the strength of an agreement between the results by the new rapid test and the new ELISA. A kappa statistic value of >0.75, 0.40 to 0.75 and <0.40 represents excellent agreement, a good to fair agreement and a poor agreement respectively (17).

[00168] ***Results***

[00169] ***Evaluation and validation of the ELISA:*** As presented in Table 6, three cutoff values at OD 0.45, OD 0.30 and OD 0.25 were applied in evaluating the performance of the ELISA. With the cutoff value set at OD 0.45, the ELISA produced the best specificity of 100% (385 of 385) but a low overall sensitivity of 60% (136 of 227) (Table 6). However, improved performance was obtained when the cutoff value was adjusted lower to OD 0.3 or OD 0.25. The ELISA, with OD 0.25 as its cutoff value, detected overall almost 12% more SARS associated samples than did with the OD 0.45 setting (Table 6). An improved delta value of 0.53 vs. 0.10 for the positive was also obtained. In a supplementary test, further 1066 samples from healthy controls were tested and a mean OD of 0.0432 ± 0.0745 was obtained. OD 0.25 was found to be equivalent to the mean OD$\pm$3SD, whereas OD 0.45 to the mean OD$\pm$5SD. The two cutoff values of OD 0.25 and OD 0.45 produced similar high specificities of 98% vs. 99.2%, 100% vs. 100% and 92% vs. 98% with the respective supplemental healthy control and the disease controls groups of non-SARS patients who suffered respiratory illness or fever (Table 6).

Table 6

	Sensitivity					Specificity				PPV*	NPV*	Delta	
	1-10	11-20	21-30	>30	Total	HD (HK)	HD (USA)	Non-SARS (respiratory)	Non-SARS (fever)			Positive	Negative
COV_{S25}	18/31	84/120	43/57	18/19	163/227	383/385	1045/1066	50/50	46/50				
	58%	70%	75%	95%	71.8%	99.5%	98.0%	100%	92%	98.8%	85.7%	0.53	2.42
COV_{S30}	16/31	78/120	42/57	18/19	155/227	384/385	1050/1066	50/50	48/50				
	52%	65%	74%	95%	68.3%	99.7%	98.5%	100%	96%	99.4%	84.2%	0.46	2.61
COV_{S35}	15/31	65/120	40/57	16/19	136/227	385/385	1058/1066	50/50	49/50				
	48%	54%	70%	84%	59.9%	100%	99.2%	100%	98%	100%	80.8%	0.10	3.03

* PPV and NPV were calculated using the results of healthy donors (Hong Kong) and those of SARS patients obtained at the same testing site in Hong Kong.

[00170] With the cutoff value set at OD 0.25, the ELISA detected IgG antibodies to SARS-CoV with not only late stage convalescent samples with a high sensitivity of 95% but also acute specimens. It detected IgG antibodies to SARS-CoV in 58%, 70% and 75% of the samples collected from SARS patients 1 to 10 days, 11 to 20 days and 21 to 30 days after the onset of clinical symptoms. The specificity obtained with the cutoff value of OD 0.25 remained to be high at 99.5% (383 of 385) with the healthy control group tested at the same testing site (Table 6). The test thus provided an overall PPV of 98.8% and NPV of 85.7%. The performance of the ELISA was also found to concord well with IF titers generated by the IF test with R^2=0.9342 (Figure 12).

[00171] ***Evaluation of the rapid immunochromatographic test:*** When the same set of clinical specimens were tested using the rapid immunochromatographic test, similar performance to that of the ELISA were obtained. The overall detection rate with SARS associated specimens by the rapid test was 70.5% (160 of 227) with its specificity at 97.7% (376 of 385) (Table 7). The respective detection rates were 55%, 68%, 81% and 79% for the four groups of samples collected from SARS patients of 1 to 10 days, 11 to 20 days, 21 to 30 days and greater than 30 days after the onset of clinical symptoms (Table 7). As this test utilized two recombinant proteins separately represented by two resultant bands, the performances of the two proteins were also analyzed. The Gst-N protein alone detected antibodies to SARS-CoV in 157 out of the 160 samples found to be positive by the two proteins combined (Table 7).

Furthermore, the Gst-N protein presented less cross-reactivity reacting to only one of the 385 specimens from the healthy controls. In contrast, the Gst-U274 detected only an overall of 8% (18 of 227) of the SARS associated samples but contributed to most of the non-specific reactivity with samples from the healthy controls (8 of 385). Interestingly, the three samples that were detected only by the Gst-U274 but not the Gst-N protein were those collected from patients at an earlier stage 1 to 20 days after the onset of clinical symptoms (Table 7). The test was therefore showed to have PPV and NPV of 94.7% and 84.9% respectively with the tested populations (Table 7). Again in a supplementary test, the rapid test was further evaluated with disease control groups of non-SARS patients who suffered respiratory illness or fever and found to cross-react to only 3 of 50 or 4 of 50 of the tested samples in the respective groups (Table 7). When the results were compared, the rapid test and the ELISA gave an excellent overall agreement of 92.5% with a kappa statistic of 0.81 (Table 8). In addition, the performance of test was also found to concord well with immunofluorescence (IF) titers generated by the IF test with R^2=0.9182 (Figure 12).

Table 7 Performance of the rapid immunochromatographic test in detecting IgG antibodies to SARS

	Sensitivity					Specificity			PPV*	NPV*
	1-10	11-20	21-30	>30	Total	HD (HK)	Non-SARS (respiratory)	Non-SARS (fever)		
Combined	17/31	82/120	46/57	15/19	160/227	376/385	47/50	46/50		
	55%	68%	81%	79%	70.5%	97.7%	94%	92%	94.7%	84.9%
Gst-N	16/31	80/120	46/57	15/19	157/227	384/385	48/50	47/50		
	52%	67%	81%	79%	69.2%	99.7%	96%	94%	99.4%	84.6%
Gst-U274	1/31	8/120	5/57	4/19	18/227	377/385	49/50	49/50		
	3%	7%	9%	21%	8%	97.9%	98%	98%	66.7%	64.3%

* PPV and NPV were calculated based on results of healthy donors and those of SARS patients obtained at the same testing site in Hong Kong

Table 8 Agreement between the immunochromatography and ELISA assay

Rapid Test	ELISA Tests		Agreement (%)	Kappa statistic[a]
	Positive	Negative		
Positive	144	25	85.2%	0.81
Negative	21	422	95.3%	

[00172] **Example 4: Western Blot as confirmatory test for diagnosis of SARS**

[00173] ***Materials and Methods***

[00174] ***Serum specimens:*** Forty convalescent serum specimens were collected with consent from SARS patients who were admitted to the Tan Tock Seng Hospital or the Singapore General Hospital. Fifty sera from healthy donors, purchased from BioClinical Partner Inc. (Franklin, Mass.) were also included in the study as healthy controls. For the non-SARS disease controls, archived Genelabs Diagnostics (Genelabs Diagnostics, Singapore) serum samples from previous studies were used and they were obtained prior to the SARS outbreak. These included 50 samples each from patients who had non-SARS related fever (confirmed as dengue fever) or suffered non-SARS related respiratory illness (confirmed as tuberculosis). In addition, 18 samples identified as false positives from screening 1066 healthy donors in a previous study (Guan et al. submitted) were also included in the present study.

[00175] ***SARS-CoV viral lysate and recombinant proteins:*** The SARS-CoV viral lysate was purchased from ZeptoMetrix Corporation (Buffalo, New York) and they were obtained from SARS CoV-infected Vero cells after sucrose gradient purification and treatment with a disruption buffer (KCl, 0.6M) containing a Triton X-100 (0.5%).

[00176] The recombinant proteins were prepared as described above. Briefly, all the proteins were expressed as GST-fusion proteins in *E. coli* but only the GST-N was purified using GSH-sepharose beads (Amersham Pharmacia). For the GST-S, the

GST-M and the GST-E proteins, the separation of the respective insoluble proteins in pellet was carried out by washing and re-suspension of the proteins and eventually by electrophoresis in 10% PAGE-SDS gels. Gel strips containing the respective GST-fusion proteins were then cut and subjected to the elution using Mini Trans-Blot™ cell (BioRad). The resulting fusion proteins were detected in Western Blot using an anti-GST monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.) and their concentrations were estimated by comparing with BSA standards in Coomassie blue-stained PAGE gel (Shen et al., submitted).

[00177] ***Mouse and Rabbit antisera*:** Specific antisera were raised by inoculating mice or rabbits as described elsewhere (21) using the respective recombinant proteins. The mouse antisera specific to the spike, the nucleocapsid, or the membrane proteins were generated by subcutaneous inoculation of BALB/c mice with 50μg of the respective recombinant proteins emulsified in complete Freund's adjuvant (Sigma, St Louis, MO). Whereas the rabbit antiserum specific to the envelope recombinant protein was generated by subcutaneous inoculation of New Zealand white rabbits with 1 mg of the purified protein again emulsified in complete Freund's adjuvant. The animals were boosted at 2 weeks interval for 8 to 16 times with the same amount of the respective purified proteins but emulsified in incomplete Freund's adjuvant (Sigma, St. Louis, MO). Sera from the immunized animals were harvested 10 days after the last immunization and were adsorbed with mammalian cell cultures to reduce unspecific binding to cellular proteins.

[00178] ***SDS-polyacrylamide gel electrophoresis (PAGE) and transblot:*** Separation of the SARS-CoV viral lysate was performed on 11% separating gels with a 3.5% stacking gel. In particular, 70 μg of the SARS-CoV viral lysate in 800 μl of denaturing buffer of 3.2% SDS, 0.5 M Tris (pH6.8), 32% glycerol, 3.2% 2-mercaptoethanol and 0.05% bromophenol blue tracking dye was boiled for 5 min in a water bath. For molecular weight determination, 50μl each of the treated lysate sample and the rainbow molecular markers were applied to separate wells (8 mm width) on the same SDS-PAGE gel. For other immunoblot analysis, the treated sample (800 μl) was applied to the preparative well (130 mm width) and electrophoresed at a constant current until the tracking dye reached the bottom. The separated proteins were electro-transferred in a tank apparatus (Hoeffer, San

Francisco, Calif.) to a nitrocellulose membrane (Whatman, Gerbershausen, Germany). After the transfer, the membranes were further deposited with goat anti-human IgG (1.6 μg per membrane, as a sample addition control) and the GST-N recombinant protein (4.3 ng per membrane) using the Autoslot™ machine (Genelabs Diagnostics, Singapore). The membrane was then incubated for 45 min in a blotting solution containing 5% nonfat milk powder before being rinsed for 30 min in PBS containing 0.5% Tween-20. Subsequently, the membrane was left to dry at room temperature (RT) for 20 min prior to being cut into 3 mm strips and stored at 2-8°C until used.

[00179] ***Western immunoblot assay and analysis:*** The Western immunoblot assay was carried out at room temperature on a rocking platform for all incubation steps. When testing, the membrane strips were placed in AutoBlot incubation trays (Genelabs Diagnostics, Singapore) and soaked for 5 min in 1 ml per strip of a Tris-based wash buffer. The buffer was aspirated and the membrane strips were then incubated for 1 hour with 10μl of the respective sera in 1 ml of blocking buffer containing 5% dry milk powder. The membrane strips were washed 3 times with 1 ml of the wash buffer per strip allowing a 5 min soak for each wash after the aspiration of the sera. The membrane strips were further incubated for 1 hour with 1ml of a conjugate of goat anti-human IgG labelled with alkaline phosphatase (Kirkegaard & Perry Laboratory Inc., Gaithersburg, MD) at a dilution of 1:1000 in the blocking buffer. After the incubation, the conjugate was removed again by aspiration and the membrane strips were washed another 3 times. This was followed by a 15-min incubation of the membrane strips with a substrate solution of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitroblue tetrazolium (NBT). The resultant protein bands were analyzed subjectively by the intensity of the bands on the strips.

[00180] For assays with the mouse or rabbit antisera, a specific dilution was used for each antiserum due to their titer differences. In particular, a 1:1,000 dilution was used for the mouse anti-S antiserum and the rabbit anti-E antiserum, whereas a 1:500 dilution was used for the mouse anti-M antiserum but a 1:100,000 for the mouse anti- N antiserum. However, the conjugates of either anti-mouse or anti-rabbit antibodies labeled with alkaline phosphatase employed for the detection of the respective antisera were used at the same dilution of 1:5000.

[00181] ***Results***

[00182] ***Identification of immunoreactive proteins of SARS CoV by Western Immunoblot analysis:*** The apparent molecular weights of the immunoreactive proteins were estimated by extrapolating a plot of the logarithm of the molecular weights versus the electrophoretic mobilities of standard proteins (Figure 13). The immunoreactive proteins seen as bands on the immunblot when assayed with a strong SARS-positive control sample (P8) included discreet, diffused and clustered ones and they were at approximately 150-kDa, 97-kDa (triplet), 45-kDa (doublet), 28-kDa (triplet), and 24-kDa (diffused) (Figure 13). When the immunoblot was tested with the mouse or the rabbit antisera raised to the specific recombinant proteins, the 150-kDa, the 45-kDa and the 24-kDa proteins were found to be associated with the spike (S), the nucleocapsid (N) and the matrix (M) proteins respectively (Figure 14). In addition, a protein not reactive with the strong SARS-positive sample but associated with the envelope (E) protein was also located at approximately 10-kDa by the rabbit anti-E antiserum (Figure 14).

[00183] ***Performance of various protein markers and the Western Immunoblot:*** When the immunoblot was tested with different sets of specimens from the SARS patients or the healthy or disease controls, distinctive banding patterns specific to samples from the SARS patients or to those from the controls were observed (Figure 15). An analysis of the band patterns showed that the two protein bands (Nh and Nl) at approximately 45-kDa associated with the N protein of SARS-CoV occurred most often reacting to all the samples from SARS patients (Figure 15, Table 9). However, although the band intensities of the two proteins were much higher with those samples from the SARS patients, both Nh and Nl were rather non-specific and also reacted to samples from the healthy or disease controls (Figure 15). For example, the Nh protein cross-reacted to 64%, 68% and 52% of the samples from the healthy, the respiratory illness and the fever controls respectively (Table 10). In fact, these N related proteins reacted to 15 out of the 18 samples (83%) previously found to be false positive by an ELISA (Table 10). In contrast, the S, the M and the GST-N recombinant protein reacted to 78%, 75% and 100% of the 40 samples from the SARS patients without any cross-reactivity with any of the controls including those identified as false positive by an ELISA (Table 10). Other highly immunoreactive proteins included the 97-kDa and the 28-kDa proteins reacting respectively to 78% and 75% of the samples from the SARS patients but only to a few

of the controls with a cross-reactivity rate of 2% to 6% (Table 10). Furthermore, a protein band at approximately 60-kDa but not presented with the strong SARS-positive control for the estimation of the molecular weight was found in the paneling study. This 60-kDa protein reacted to a portion of all the tested samples (11-34%) rather non-discriminatory regardless of whether they were from the SARS patients or the healthy or disease controls (Table 10). The E protein as identified by the rabbit antisera, on the other hand, showed no immunoreactivity to any of the human serum samples tested in the study (Table 10).

Table 9 Sensitivity and specificity of the Western immunoblot

	SARS		Healthy Donors		Non-SARS (TB)		Non-SARS (fever)		ELISA (FP)	
	(n=30)		(n=30)		(n=50)		(n=50)		(n=18)	
Combination*	Positive	Sens	Positive	Spec	Positive	Spec	Positive	Spec	Positive	Spec
N+S	31	78%	0	100%	0	100%	0	100%	0	100%
N+97-kDa	31	78%	0	100%	0	100%	0	100%	0	100%
N+69-kDa	9	23%	2	90%	4	92%	0	100%	1	94%
N+28-kDa	30	75%	0	100%	0	100%	0	100%	0	100%
N+M	30	75%	0	100%	0	100%	0	100%	0	100%
N+E	0	0%	0	100%	0	100%	0	100%	0	100%
N+GST-N	30	100%	0	100%	0	100%	0	100%	0	100%

*A test result was considered positive only when the nucleocapsid (both N_1 and N_2) and the respective protein were detected simultaneously.

Table 10 Reactivity patterns of immunoreactive proteins in the Western immunoblot

	SARS		Healthy Donors		Non-SARS (TB)		Non-SARS (fever)		ELISA (FP)	
	(n=40)		(n=50)		(n=50)		(n=50)		(n=18)	
Markers	Positive	Percentage	Positive	Percentage	Positive	Percentage	Positive	Percentage	Positive	Percentage
S	31	78%	0	0%	0	0%	0	0%	0	0%
97-kDa*	31	78%	2	4%	1	2%	1	2%	0	0%
60-kDa	9	23%	11	22%	17	34%	11	22%	2	11%
N_h	40	100%	32	64%	34	68%	26	52%	15	83%
N_l	40	100%	21	42%	17	34%	17	34%	13	72%
28-kDa*	30	75%	0	0%	0	0%	3	6%	0	0%
M	30	75%	0	0%	0	0%	0	0%	0	0%
E	0	0%	1	2%	0	0%	0	0%	0	0%
GST-N	40	100%	0	0%	0	0%	0	0%	0	0%

* Number as any one of the three bands in the region appeared

[00184] A further analysis of the band patterns of the immunoreactive proteins revealed useful criteria for differentiating samples from the SARS patients and those from the controls. When simultaneous detection of the N proteins (both Nh and Nl) and the S protein was set as a prerequisite for a test result to be interpreted as positive for SARS, the immunoblot was found to have a sensitivity of 78% with a specificity of 100% to all four control groups (Table 9). Similarly, when the N proteins (again, both Nh and Nl) were considered with the 97-kDa, the 28-kDa, the M or the GST-N protein respectively for result interpretation, the immunoblot assay was found to have a sensitivity of 78%, 75%, 75% and 100% respectively, while maintaining a specificity of 100% (Table 9). Both the 60-kDa and the E protein, however, were found to be less useful in any combination due to the lack of sensitivity (Table 9). A combined interpretation criterion utilizing the N proteins and at least one of the specific proteins including, the S, the 97-kDa, the 28-kDa, and the M proteins provided a kit sensitivity of 95% and specificity of 100% (Table 11). Further addition of the GST-N recombinant protein to the above interpretation criterion improved the sensitivity to 100% without altering the specificity (Table 11).

Table 11 Sensitivity and specificity of the Western immunoblot based on set criteria

	SARS		Healthy Donors		Non-SARS (TB)		Non-SARS (fever)		ELISA (FP)	
	(n=40)		(n=50)		(n=50)		(n=50)		(n=18)	
Criteria*	Positive	Sens	Positive	Specs	Positive	Specs	Positive	Specs	Positive	Specs
Combination 1	38	95%	0	100%	0	100%	0	100%	0	100%
Combination 2	40	100%	0	100%	0	100%	0	100%	0	100%

*For combination 1, a test result was considered positive only when the nucleocapsid (both N_R and N_I) and at least one of the lysate proteins, i.e. the S, the 97-kDa, the 28-kDa, or the M were detected simultaneously. For combination 2, a test result was considered positive only when the nucleocapsid (both N_R and N_I) and at least one of the lysate proteins, or the GST-N recombinant protein were detected simultaneously.

[00185] As can be understood by one skilled in the art, many modifications to the exemplary embodiments described herein are possible. The invention, rather, is intended to encompass all such modification within its scope, as defined by the claims.

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WHAT IS CLAIMED IS:

1. A method for detecting the presence or absence of antibody to SARS coronavirus (CoV), the method comprising the step of contacting a SARS CoV antigen with an antibody-containing sample, for a time and under conditions sufficient for the antibody to form a complex with the antigen, wherein the antigen comprises a protein selected from the group consisting of S, M, E, N, U274 and an immunogenic fragment thereof, followed by the step of detecting the complex by contacting the complex with an anti-human IgA antibody or an anti-human IgM antibody, and wherein detection of the specific binding between the antibody and the protein complex indicates that the sample contains antibody specific to SARS CoV.

2. The method of claim 1 for testing whether a human is infected by or has been exposed to SARS CoV, wherein the antibody-containing sample is from the human being tested, and wherein specific binding between the antibody and the protein indicates that the human is infected by or has been exposed to SARS CoV.

3. The method of claim 1 or claim 2 wherein the antigen comprises a protein selected from the group consisting of M, E, N, U274 and an antigenic fragment thereof.

4. The method of claim 1 or claim 2 wherein the antigen comprises:
amino acids 46 to 480 of S,
N having amino acids 111 to 118 deleted, or
amino acids 134 to 274 of U274.

5. The method of claim 3 wherein the antigen comprises:
N having amino acids 111 to 118 deleted, or
amino acids 134 to 274 of U274.

6. The method of any one of claims 1 to 3 wherein the antigen comprises N or U274 or an immunogenic fragment thereof.

7. The method of any one of claims 1 to 6 wherein the protein is fused to a heterologous protein.

8. The method of claim 7 wherein the heterologous protein is glutathione S transferase (GST).

9. The method of any one of claims 1 to 8 wherein the antigen is immobilized.

10. The method of any one of claims 3 to 9 wherein the sample is serum.

11. The method of claim 10 wherein the serum is collected from a human who was infected by or has been exposed to SARS CoV, between 2 and 60 days post-infection.

12. The method of claim 10 wherein the serum is collected from a human who was infected by or has been exposed to SARS CoV, between 2 and 11 days post-infection.

13. The method of claim 10 wherein the serum is collected from a human who was infected by or has been exposed to SARS CoV, between 16 and 60 days post-infection.

14. The method of any one of the preceding claims wherein the step of detecting the complex comprises visualizing the complex by means of an enzyme, a coloured dye, a fluorescent dye, a chemiluminescent molecule, a molecule containing a radioactive atom or a molecule containing a heavy metal.

15. The method of claim 14 wherein the enzyme is alkaline phosphatase or horseradish peroxidase, wherein the fluorescent dye is FITC or rhodamine, and wherein the molecule containing a heavy metal is colloidal gold complex.

16. An isolated antibody directed against an antigenic SARS CoV protein, wherein the antigenic SARS CoV protein is S, M, E, N or U274 protein or an antigenic fragment thereof detectable by contacting a complex of the antibody and the

antigenic SARS Co V protein with an anti-human IgA antibody or an anti-human IgM antibody.

17. The antibody of claim 16 wherein the antibody is directed against M, E, N or U274 protein or an antigenic fragment thereof.

18. The antibody of claim 16 wherein the antibody is directed against
amino acids 46 to 480 of S,
N having amino acids 111 to 118 deleted, or
amino acids 134 to 274 of U274.

19. The antibody of claim 17 wherein the antibody is directed against
N having amino acids 111 to 118 deleted, or
amino acids 134 to 274 of U274.

20. The antibody of any one of claims 16 to 19 that is a polyclonal antibody.

21. The antibody of any one of claims 16 to 19 that is a monoclonal antibody.

22. A commercial package for detecting the presence or absence of antibody to SARS coronavirus (CoV) in a sample, the package comprising

(a) a SARS CoV antigen comprising a protein selected from the group consisting of S, M, E, N, U274, and an immunogenic fragment thereof; and

(b) means for detecting a complex between the antibody from the sample and the antigen comprising an anti-human IgA antibody or an anti-human IgM antibody.

23. A commercial package for testing whether a human is infected by or has been exposed to SARS coronavirus (CoV), the package comprising

(a) a SARS CoV antigen comprising a protein selected from the group consisting of S, M, E, N, U274, and an immunogenic fragment thereof; and

(b) means for detecting a complex between the antigen and an antibody against the antigen comprising an anti-human IgA antibody or an anti-human IgM

antibody, wherein the sample is an antibody-containing sample of the human being tested.

24. The package of claim 22 or claim 23 wherein the antigen comprises a protein selected from the group consisting of M, E, N, U274, and an immunogenic fragment thereof.

25. The package of claim 22 or claim 23 wherein the antigen comprises:
amino acids 46 to 480 of S,
N having amino acids 111 to 118 deleted, or
amino acids 134 to 274 of U274.

26. The package of claim 24 wherein the antigen comprises:
N having amino acids 111 to 118 deleted, or
amino acids 134 to 274 of U274.

27. The package of claim 22 or claim 23 wherein the antigen comprises N or U274 or an immunogenic fragment thereof.

28. The package of any one of claims 22 to 27 wherein the protein is fused to a heterologous protein.

29. The package of claim 28 wherein the heterologous protein is glutathione S transferase (GST).

30. The package of any one of claims 22 to 29 wherein the antigen is immobilized.

31. The package of any one of claims 22 to 30 wherein the sample is serum.

32. The package of claim 31 wherein the serum is collected from a human who was infected by or has been exposed to SARS CoV, between 2 and 60 days post-infection.

33. The package of claim 32 wherein the serum is collected from a human who was infected by or has been exposed to SARS CoV, between 2 and 11 days post-infection.

34. The package of claim 32 wherein the serum is collected from a human who was infected by or has been exposed to SARS CoV, between 16 and 60 days post-infection.

35. The package of any one of claims 26 to 34 wherein the means for detecting the complex further comprises a visualizing means selected from the group consisting of an enzyme, a coloured dye, a fluorescent dye, a chemiluminescent molecule, a molecule containing a radioactive atom and a molecule containing a heavy metal.

36. The package of claim 35 wherein the enzyme is alkaline phosphatase or horseradish peroxidase, wherein the fluorescent dye is FITC or rhodamine, and wherein the molecule containing a heavy metal is colloidal gold complex.

37. A method of detecting the presence or absence of S, M, E, N or U274 SARS CoV protein, the method comprising contacting an antibody directed against a protein selected from the group consisting of S, M, E, N and U274, with an antigen-containing sample, for a time and under conditions sufficient for the antibody to form a complex with an antigen followed by the step of detecting the complex by contacting the complex with an anti-human IgA antibody or an anti-human IgM antibody; and wherein detection of the specific binding between the antibody and the antigen complex indicates that the sample contains an antigen that comprises S, M, E, N, U274 or an immunogenic fragment thereof.

38. A method of detecting the presence or absence of M, E, N or U274 SARS CoV protein, the method comprising contacting an antibody directed against a protein selected from the group consisting of M, E, N and U274, with an antigen-containing sample, for a time and under conditions sufficient for the antibody to form a complex with an antigen followed by the step of detecting the complex by contacting the

complex with an anti-human IgA antibody or an anti-human IgM antibody; and wherein detection of the specific binding between the antibody and the antigen complex indicates that the sample contains an antigen that comprises M, E, N, U274 or an immunogenic fragment thereof.

39. The method of any one of claims 37 or 38 that is a Western immunoblot, ELISA, capture ELISA or immunofluorescence assay.

40. The method of any one of claims 37 to 39 wherein the step of detecting the complex comprises visualizing the complex by means of an enzyme, a coloured dye, a fluorescent dye, a chemiluminescent molecule, a molecule containing a radioactive atom or a molecule containing a heavy metal.

41. The method of claim 40 wherein the enzyme is alkaline phosphatase or horseradish peroxidase, wherein the fluorescent dye is FITC or rhodamine, and wherein the molecule containing a heavy metal is a colloidal gold complex.