DIAGNOSTIC METHODS

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates generally to a method of detecting exposure of a subject such as an animal and in particular a mammal and even more particularly a human to a coronavirus or immunological relative thereof. More particularly, the present invention relates to screening of a biological sample taken from a subject such as a mammal including a human for the presence of binding partners of coronavirus-derived components in order to determine if the subject is infected with or has been exposed to a coronavirus or immunological relative thereof. The present invention further provides diagnostic kits for the detection of a present or prior exposure of a subject to a coronavirus or immunological relative thereof. The ability to rapidly diagnose the presence of a coronavirus or its relatives enables medical or veterinary intervention and treatment at an early stage to prevent or reduce the risk of spread of infection.

DESCRIPTION OF THE PRIOR ART

Bibliographic details of the publications referred to in this specification are also collected at the end of the description.

Reference to any prior art in this specification is not, and should not be taken as an acknowledgement or any form of suggestion that this prior art forms part of the common general knowledge in any country.

The recent outbreaks of severe acute respiratory syndrome (SARS) in China, Hong Kong, Taiwan, Canada, Vietnam and Singapore led to the discovery of a novel coronavirus (CoV) which was identified as the probable causative agent of SARS (Peiris *et al., Lancet 361*: 1319-1325, 2003; Ksiazek *et al., N Engl J Med 15;348(20)*: 1953-66, 2003; Drosten *et al., N Engl J Med 15;348(20)*: 1967-76, 2003). Although human coronaviruses cause up to 30 percent of colds, previously they had rarely been associated as causing diseases of the lower respiratory tract.

It was also previously thought that coronaviruses only caused disease in one host species, however, the emergence of SARS lead scientists to speculate that either the SARS-associated CoV could have arisen as a mutant of a human coronavirus that acquired new virulence factors, as a mutant of an animal coronavirus that can infect human cells, or as a recombinant of two human coronaviruses or a human coronavirus and an animal coronavirus. Studies to determine the evolution of SARS demonstrate that antibodies to the SARS-associated CoV were found in serum samples obtained from patients with SARS during convalescence but not in human serum samples banked prior to the SARS outbreak. This finding suggested that the SARS-associated CoV was new to the human population. Further studies showed that the nucleotide sequence of the SARS-associated CoV genome differed substantially from sequences of all known coronaviruses, suggesting that the SARS-associated CoV was neither a mutant of any known coronavirus nor a recombinant of known coronaviruses. Taken together, these findings suggested that the SARS-associated CoV is a previously unknown coronavirus, probably from a non-human host, that somehow acquired an ability to infect humans.

With the emerging realization that new viruses, such as the CoV which caused SARS, are able to infect humans and be rapidly spread across the world, there is a heightened awareness of a need for the rapid development of diagnostic methods which are able to quickly and easily detect the presence of a coronavirus or immunological relatives thereof in an infected subject.

Since the outbreak of SARS, several reverse transcriptase-polymerase chain reaction (RT-PCR) assays, which detect specific sequences of the SARS CoV RNA genome have been developed for use in diagnostic protocols. As with most PCR based assays, they are very sensitive and laboratory studies have indicated that they are able to identify very low copy numbers of the viral genome present in the biological sample. The ideal clinical specimen in which to detect SARS CoV genome is respiratory secretions, either in the form of throat swabs or nasopharyngeal aspirates. However, procedures to collect such specimens often induce coughing leaving healthcare workers at risk of being infected with CoV and acquiring SARS from their patients. In addition, such assays require technologically sophisticated and cumbersome machinery.

Alternative diagnostic assays for CoV and SARS are, therefore, needed. Serological detection procedures have the potential to play a role in clinical diagnosis to confirm or exclude the diagnosis of SARS-associated CoV.

SUMMARY OF THE INVENTION

Throughout the specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

The present invention is predicted in part on a need to develop a low cost, rapid and straight forward assay to determine present or prior exposure to SARS-associated CoV. In accordance with the present invention, subjects including animals, such as mammals and in particular humans are screened for the presence of binding partners of CoV components. The preferred binding partners are subject-derived binding partners such as, but not limited to, immunointeractive molecules. The most preferred immunointeractive molecules are antibodies. Other binding partners include T-cell associated binding molecules (TABMs) or a nucleic acid molecule complex comprising same (e.g. RNAi) or any other subject-derived molecule, which bind specifically to a CoV-derived component. The identification of such binding partners is then used as evidence of present or prior exposure of a subject CoV.

Accordingly, one aspect of the present invention contemplates a method for detecting exposure of a subject to a coronavirus or immunological relative thereof, said method comprising the steps of:

(a) contacting a biological sample from the subject with a component derived from the coronavirus or immunological relative thereof for a time and under conditions sufficient to facilitate the formation of a complex between the component and a subject-derived binding partner of the component contained within the biological sample; and

(b) determining the presence of the complex, wherein the presence of the complex is indicative of exposure of the subject to a coronavirus or immunological relative thereof.

As indicated above, the preferred subject-derived binding partner is an antibody or other immunointeractive molecule.

Consequently, another aspect of the present invention provides a method for detecting exposure of a subject to a coronavirus or immunological relative thereof, said method comprising the steps of:

(a) contacting a biological sample from the subject with a component derived from the coronavirus or immunological relative thereof for a time and under conditions sufficient to facilitate the formation of a complex between the component and subject derived immunointeractive molecule contained within the biological sample; and

(b) determining the presence of the complex, wherein the presence of the complex is indicative of exposure of the subject to a coronavirus or immunological relative thereof.

Reference to "steps" is not to be taken in any defined order and the "steps" may in fact occur concurrently.

In another aspect, the present invention provides kits for the detection of a coronavirus or immunological relative thereof, wherein the kits contain:

(a) a solid support;

(b) a component derived from or a total or partial extract of a coronavirus or immunological relative thereof;

(c) an incubation plate comprising at least 1 well;

(d) blocking buffer;

(e) washing buffer;

(f) at least one binding partner such as an immunointeractive molecule conjugated to a reporter molecule capable of providing a detectable signal; and optionally

(g) instructions for using the kit.

The method and kit of the present invention allow for the detection and diagnosis of a coronavirus or immunological relative thereof. In a preferred aspect, the coronavirus detected by the method and kit of the present invention include coronaviruses or immunological relatives thereof which cause symptoms associated with a lower respiratory tract infection. In a most preferred aspect, the method and kit of the present invention, detect exposure to a CoV which causes SARS or symptoms associated with SARS.

The method and kit of the present invention enables rapid and relatively inexpensive detection of CoV or other coronaviruses in individual subjects or populations of subjects which in turn enables isolation procedures to be implemented quickly to control spread of further infection to other subjects. Early detection further enables early therapeutic intervention.

Whilst CoV is a term used to refer to the SARS-associated coronavirus in humans, the present invention is applicable to detecting coronaviruses in any subject including non-human animals, mammals and avian species. Human subjects, however, are most preferred in accordance with the present invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is the analysis of both SARS CoV IgG positive and negative sera using the DB-EIA protocol.

Figure 2 is a comparative analysis of DB-EIA, IFA and ELISA.

Figure 3 is the result of 10 pairs of serum samples from SARS confirmed cases and their comparisons with IFA and ELISA.

Figure 4 is a graphical representation of the comparison of sensitivities between DB-EIA, ELISA and IFA.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is predicated, in part, on the identification of a method for detecting present or prior exposure of a subject to a coronavirus or immunological relative thereof. More particularly, the invention relates to serological analysis of a biological sample from a subject in order to determine the presence of a binding partner specific and discriminatory for a component derived from a coronavirus or an immunological relative thereof. The method of the present invention is useful for detecting exposure to a coronavirus or immunological relative thereof which causes symptoms associated with lower respiratory tract infections. In a preferred aspect, the method of the present invention enables detection of a present or a prior exposure of a subject to a CoV which causes SARS or symptoms associated with SARS.

Accordingly, one aspect of the present invention contemplates a method for detecting exposure of a subject to a coronavirus or immunological relative thereof, said method comprising the steps of:

(a) contacting a biological sample from the subject with a component derived from the coronavirus or immunological relative thereof for a time and under conditions sufficient to facilitate the formation of a complex between the component and a subject-derived binding partner of the component contained within the biological sample; and

(b) determining the presence of the complex, wherein the presence of the complex is indicative of exposure of the subject to a coronavirus or immunological relative thereof.

In a preferred embodiment the binding partner is an interactive molecule such as an antibody, TABM or nucleic acid based molecule such as RNAi. Most preferably, however, the binding partner is an immunointeractive molecule. In a particularly preferred embodiment, the immunointeractive molecule is an antibody.

Accordingly, another aspect of the present invention is a method for detecting exposure of a subject to a coronavirus or immunological relative thereof, said method comprising the steps of:

(a) contacting a biological sample from the subject with a component derived from the coronavirus or immunological relative thereof for a time and under conditions sufficient to facilitate the formation of a complex between the component and a subject derived-immunointeractive molecule contained within the biological sample; and

(b) determining the presence of the complex, wherein the presence of the complex is indicative of exposure of the subject to a coronavirus or immunological relative thereof.

In describing or claiming the present invention, the following terminology is used in accordance to the definitions set forth below.

It is to be understood that unless otherwise indicated, the subject invention is not limited to specific formulations of components, manufacturing methods or the like, and as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

It must be noted that, as used in the subject specification, the singular forms "a", "an", and "the" include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to an "immunointeractive molecule" includes a single immunointeractive molecule, as well as two or more immunointeractive molecules; reference to a "coronavirus" includes a single coronavirus or two or more coronaviruses or species of coronaviruses, and so forth.

A coronavirus is defined as a group of RNA animal viruses consisting of enveloped particles 80-120 nm long, with helical capsids. These viruses are named for their corona-like (halo) appearance in electron micrographs. The corona or halo is due to an array of surface projections on the viral envelope, one of which is the E2 glycoprotein, the viral attachment protein and target of neutralizing antibodies. Coronaviruses are second only to rhinoviruses as a cause of the common cold. Coronavirus infect epithelial cells and remains localized in the upper respiratory tract, due to the optimum growth temperature of the virus, which is 33° to 35°C. Coronavirus particles have been seen in electron micrographs of the stools of children and adults with diarrhea and gastroenteritis. However, an association with these diseases has never been proven.

Coronaviruses infect a variety of mammals and birds. The exact number of human isolates is not known, as many cannot be grown in culture. They are known to cause *inter alia*:

- respiratory infections;
- enteric infections; and
- and possibly neurological syndromes.

Transmitted by aerosols of respiratory secretions, growth appears to be localized in epithelium of upper respiratory tract, but there is no adequate animal model for the human respiratory coronaviruses. Clinically, most infections cause a mild, self-limited disease (classical 'cold' or upset stomach), but there may be rare neurological complications.

Therefore, the present invention is directed to detecting exposure to coronaviruses such as SARS-associated coronaviruses and in particular CoV which infect humans. Examples of particular CoV strains include HCoV-229E, HCoV-OC43 and SARS-CoV. Other coronaviruses contemplated herein include TGEV, PRCoV, PEDV, HEV, all of which infect pigs, FIPV, FcoV, both of which infect cats, CcoV, which infects dogs, RcoV, which infects rats, MHV, which infects mice, BcoV, which infects cattle and IBV and TcoV, which infect chickens and turkeys, respectively.

In a related aspect, the method of the present invention is able to detect exposure to immunological relatives of a coronavirus. As used herein an "immunological relative" is defined as any virus which carries an antigenic epitope which cross-reacts with an antibody directed to a coronavirus component or which elicits an immunological response which cross-reacts with a coronavirus.

Viruses detected by the method of the present invention include mutants of a human coronavirus, mutants of an animal coronavirus, or viruses that have arisen due to a recombination of two human coronaviruses or a human coronavirus and an animal coronavirus or even two animal or two avian or an animal and an avian coronavirus and which has then crossed the species barrier to enable infection of humans. Additionally, virus exposure detected by the method of the present invention include exposure to hybrids of animal or avian and humans viruses which have arisen due to the recombination of a coronavirus or coronaviruses and another class of virus. Other viruses include, but are not limited to, double-stranded DNA viruses, single-stranded DNA viruses, (+) single-stranded RNA viruses, (-) single-stranded RNA viruses, double-stranded DNA viruses, (+) single-stranded RNA retroviruses, human immunodeficiency virus (*HIV*), the human papilloma virus, Epstein-Barr virus, the polio virus, the rabies virus, the Ebola virus, the influenza virus, the encephalitis virus, smallpox virus, the rabies virus, the herpes viruses, the sendai virus, the respiratory syncytial virus, the othromyxoviruses, the measles viruses, the vesicular stomatitis virus, visna virus and cytomegalovirus.

As used herein, a "biological sample" is any sample from a subject which contains a subject-derived putative binding partner or a coronavirus component. Preferably, the biological sample comprises immunointeractive molecules specific or discriminatory of a coronavirus component. A biological sample may be obtained therefore from any source of material which contains immune material. Preferably, the biological sample used in the method of the present invention is derived from human subject although the present invention extends to screening a range of non-human subjects.

Reference to a "subject" should be understood as a reference to a human or non-human

animal or avian species. Non-human subjects contemplated by the present invention include non-human primates, livestocks (e.g. sheep, pigs, cows, horses, goats, donkeys), laboratory animals (e.g. mice, hamsters, rabbits, rats, guinea pigs), domestic companion animals (e.g. dogs, cats), captive wild or tamed animals (e.g. foxes, kangaroos, dingoes), reptiles or fish. Avian species include any bird (e.g. chicken, geese, ducks and other poultry birds, game birds, emus, ostriches).

It should be understood that the biological sample may be any sample of material derived from the subject. This includes reference to both samples which are naturally present in the subject, such as tissue and body fluids (for example biopsy specimens such as lymphoid specimens, blood, lymph fluid, sputum, respiratory including nasal fluid, feces or bronchial secretions) and samples which are introduced into the body of the subject and subsequently removed, such as, for example, the saline solution extracted from the lung following a lung lavage or from the colon following an enema.

The biological sample which is tested according to the method of the present invention may be tested directly or may require some form of treatment prior to testing. For example, a blood sample may be processed in order to facilitate the removal of all cellular material. Such processing may be the centrifugation of the blood in order to pellet the cellular component. Such techniques, which could include centrifugation, would be familiar to those of skill in the art. Filtration and clotting methods may also be employed.

As indicated above, reference to "steps" in the subject method is not to be taken as a defined order and two or more steps may be combined or concurrently practiced.

"Detecting" the formation of a complex between a component derived from a coronavirus or an immunological relative thereof and a subject-derived binding partner such as an immunointeractive molecule, may be by any convenient method which will be known to those of skill in the art. In one aspect, a secondary antibody which is conjugated to a label is added to the complex to facilitate detection. A range of labels providing a detectable signal may be employed. The label may be selected from a group

including a chromogen, a catalyst, an enzyme, a fluorophore, a luminescent molecule, a chemiluminescent molecule, a lanthanide ion such as Europium (Eu^{34}), a radioisotope and a direct visual label. In the case of a direct visual label, use may be made of a colloidal metallic or non-metallic particular, a dye particle, an enzyme or a substrate, an organic polymer, a latex particle, a liposome, or other vesicle containing a signal producing substance and the like. A large number of enzymes suitable for use as labels are disclosed in United States Patent Nos. 4,366,241, 4,843,000 and 4,849,338. Suitable enzyme labels useful in the present invention include alkaline phosphatase, horseradish peroxidase, luciferase, β-galactosidase, glucose oxidase, lysozyme, malate dehydrogenase and the like. The enzyme label may be used alone or in combination with a second enzyme which is in solution. Alternatively, a fluorophore which may be used as a suitable label in accordance with the present invention includes, but is not limited to, fluorescein-isothiocyanate (FITC), and the fluorochrome is selected from FITC, cyanine-2, Cyanine-3, Cyanine-3.5, Cyanine-5, Cyanine-7, fluorescein, Texas red, rhodamine, lissamine and phycoerythrin. The detection of the complex of the present invention is preferably achieved by the addition of a secondary antibody attached to horseradish peroxidase, which when reacted with its substrate, DAB, produces a visually detectable color change.

As indicated herein, the preferred binding partner is an immunointeractive molecule.

Reference to an "immunointeractive molecule" should be understood as a reference to any molecule comprising an antigen binding portion or a derivative thereof. Preferably, the immunointeractive molecule is an antibody produced during a humoral response in the subject to coronavirus infection.

As used herein, reference to a "component derived from a coronavirus or immunological relative thereof" refers to a substance on, in or encoded by a coronavirus or its immunological relative thereof which either elicits a response in the subject resulting in the production of a binding partner such as an antibody. In a preferred embodiment, the component derived from the coronavirus or immunological relative thereof is a viral protein or immunogenic fragment thereof, a glycoprotein, a lipid,

carbohydrate, DNA or RNA or combination thereof such as a lipoprotein, phospholipid or phospholipoproetin. Viral proteins contemplated by the present invention include:

Small Envelope Protein:

This protein is located at genome position: 26,117-26,347. The small envelope protein has a predicted length of 76 amino acids. The predicted structure for this protein is a Type II membrane protein with the majority of the hydrophilic domain (46 residues) and the C terminus located outside the viral particle.

Orf3

Orf3 is a protein with a predicted length of 274 amino acids. The predicted structure comprises C-terminus and the adjacent 149-amino acid region lie inside the viral membrane. It is also thought that the C-terminal region may have ATP-binding abilities.

Spike (S) Glycoprotein (E2)

The SARS spike glycoprotein is a surface projection glycoprotein precursor, with a predicted length of 1,255 amino acids. The spike protein is conserved among other coronaviruses, and it is believed that three molecules of the spike protein form the characteristic structure observed in these viruses. The evidence gives an overall model of a type I membrane protein with the N-terminus and the majority of the protein (residues 14-1195) on the outside of the virus particle.

Orf14

The predicted length of Orf14 is 70 amino acids.

Orf10

Orf10 has a predicted length of 39 amino acids.

Orf9

Orf9 has a predicted length of 44 amino acids. The evidence points to a type II membrane protein.

Orf8

Orf8 has a predicted length of 122 amino acids. The membrane prediction evidence from several programs points to a type I membrane protein with the N-terminus and a major hydrophilic domain (residues 16-98) on the surface of the viral particle.

Orf7

Orf7 has a predicted length of 63 amino acids.

Orf4

Orf4 has a predicted length of 154 amino acids. It overlaps completely with Orf3 and the small envelope protein.

Membrane (Matrix) Glycoprotein M

The membrane glycoprotein M has a predicted length of 221 amino acids. Analysis revealed similarity to many coronaviral matrix proteins. The M protein is known to interact with the S (Spike) glycoprotein, as well as the N protein, during formation of the viral envelope and viral assembly in other coronaviruses.

Replicase 1A/B

The replicase 1A Orf extends from base 265 to 13,398; the 1B Orf extends from base 13,398 to 21,485. As in other coronaviruses, a frame shift separates the 1a and 1b reading frames. The replicase is highly similar in both length and amino acid sequence to the replicase proteins found in other coronaviruses. The genes code several proteins that are produced by proteolytic cleavage of the large initial polyprotein.

Orf11

Orf11 has a predicted length of 84 amino acids. It exhibits some very short regions of similarity to a section of the human coronavirus E2 glycoprotein precursor.

Nucleocapsid Protein

The nucleocapsid protein of SARS bears a high similarity to the nucleocapsid proteins of other coronaviruses. However, it contains a short unique lysine-rich region

(KTFPPTEPKKDKKKKTDEAQ) that is not found in any other viruses. It is speculated that it may be a novel nuclear function involved in pathogenesis. The highly basic nature of the peptide is characteristic of an RNA-binding protein.

Orf13

The predicted length of Orf13 is 98 amino acids.

As indicated herein the preferred binding partner is an antibody produced in the subject to a coronavirus or related virus component. However, a binding partner of the targeted antibody may also be used. An example of such a binding partner is an anti-idiotypic antibody or an antibody specific for and discriminatory of a subject-derived antibody specific for a coronavirus or related virus component.

As used herein, an "anti-idiotypic antibody" is an antibody which binds to the specific antigen binding site of another antibody generated in response to exposure to a component derived from a coronavirus or immunological relative thereof.

An immune response, as used herein, refers to a selective response mounted by the immune system of vertebrates in which specific antibodies and/or cytotoxic cells are produced against invading coronaviruses or immunological relatives thereof, which are recognized as foreign by the body. The production of antibodies circulating in the blood is known as a humoral immune response, the production of cytotoxic cells as a cell-mediated or cellular immune response

The preferred method of the present invention relies upon the detection of a complex formed between an immunointeractive molecule contained within a biological sample and a component derived from a coronavirus or an immunological relative thereof. A complex may comprise one or more immunointeractive molecules bound to one or more components derived from a coronavirus or an immunological relative thereof.

Reference to a biological sample being placed in contact with a component derived from a coronavirus or an immunological relative thereof should be understood as a

reference to any method of facilitating the interaction of one or more immunointeractive molecules of the biological sample with a component derived from a coronavirus or an immunological relative thereof such that coupling, binding or otherwise association between the immunointeractive molecule and a specific component derived from a coronavirus or an immunological relative thereof can occur. For example, the component derived from a coronavirus or an immunological relative thereof may be bound to a membrane, such as a nitrocellulose membrane, or to any solid support, and the biological sample added. The biological sample is then left in contact with the bound component derived from a coronavirus or an immunological relative thereof for a period of time sufficient and under conditions which allow the stable formation of a complex. Once a complex is formed, a detection system is then added to facilitate the detection of the complex. In yet another aspect, the biological sample may be applied to a membrane, such as a nitrocellulose membrane, or to a solid support, and the component derived from a coronavirus or an immunological relative thereof added. The component derived from a coronavirus or an immunological relative thereof is then left in contact with the biological sample for a period of time sufficient and under conditions which allow the stable formation of a complex. Once a complex is formed, a detection system is then added to facilitate the detection of the complex.

Solid supports contemplated for use in the method of the present invention include any support to which either the immunointeractive molecules or components derived from the coronavirus or immunological relative thereof can be bound. Examples of solid supports contemplated by the method and kit of the present invention, include, without being limited to, membranes, beads, plates, wells, microspheres and microparticles.

Membranes contemplated by the method and kit of the present invention include any membrane to which either the immunointeractive molecules or components derived from the coronavirus or immunological relative thereof can bind. Examples of membranes, include without being limited to, nitrocellulose membranes, polyamide membrane filters, polycarbonate membrane filters, regenerated cellulose membrane filters, polytetrafluorethylene (PTFE) membrane filters, cellulose acetate membrane filters and cellulose nitrate membrane filters with filter paper carrier.

The method and kit of the present invention can detect exposure of an animal to a coronavirus or immunological relative either during periods of acute infection or chronic infection. As used herein, "acute infection" refers to the period of time when a virus has infected a host and is actively replicating and/or causing symptoms associated with infection. Chronic infection refers to the period of infection, wherein the virus is no longer present or is present in dormant form or is replicating at a level which does not result in the presence of symptoms which are typically associated with acute viral infection. Using the method and kit of the present invention, exposure can be detected at any time after the generation of an immunointeractive molecule in the infected animal.

The present invention further provides a kit for detecting exposure of a subject to a coronavirus or its immunological relative. The kit may be in any convenient form but generally comprises a solid support such as described herein adapted to receive or comprise isolated components or extracts of a coronavirus or an immunological relative thereof. The kit may also comprise reagents, reporter molecules capable of providing detectable signals and optionally instructions for us. The kit may be in modular form wherein individual components may be separately purchased.

Accordingly, another aspect of the present invention is directed to a modular kit comprising one or more members wherein at least one member is a solid support comprising a component or an extract comprising a component of or derived from a coronavirus or immunological relative thereof.

In an alternative embodiment, the solid support comprises an array of binding partners for one or more components of one or more coronaviruses or immunological relative thereof from one or more subjects.

The kit may optionally be adapted to be analyzed electronically or spectrophotometrically or fluorometrically and may further be adapted fir high throughput screening.

The present invention also provides a method for assessing the relative risk of one or

more subjects being exposed to a coronavirus or immunological relative thereof within a defined location (e.g. a geographical area, industrial or housing estate, a means of transport or center for medical treatment or assessment), comprising:

(a) obtaining samples from a representative population within defined location; and

(b) assessing evidence of exposure of individual members of sample population to a coronavirus or immunological relative thereof by the method comprising the steps of:

(i) contacting a biological sample from the subject with a component derived from the coronavirus or immunological relative thereof for a time and under conditions sufficient to facilitate the formation of a complex between the component and a subject-derived binding partner of the component contained within the biological sample; and

(ii) determining the presence of the complex, wherein the presence of the complex is indicative of exposure of the subject to a coronavirus or immunological relative thereof,

and then assessing the relative risk of exposure within the defined location.

In a preferred embodiment, the present invention also provides a method for assessing the relative risk of one or more subjects being exposed to a coronavirus or immunological relative thereof within a defined location (e.g. a geographical area, industrial or housing estate, a means of transport or center for medical treatment or assessment), comprising:

(a) obtaining samples from a representative population within defined location; and

(b) assessing evidence of exposure of individual members of sample population to a coronavirus or immunological relative thereof by the method comprising the steps of:

(i) contacting a biological sample from the subject with a component derived from the coronavirus or immunological relative thereof for a time and under conditions sufficient to facilitate the formation of a complex between the component

and subject derived immunointeractive molecule contained within the biological sample; and

(ii) determining the presence of the complex, wherein the presence of the complex is indicative of exposure of the subject to a coronavirus or immunological relative thereof,

and then assessing the relative risk of exposure within the defined location.

Risk analysis may be conducted using software in a computer readable form. Consequently, the present invention further relates to a computer readable program and computer comprising same suitable for analyzing exposure of a subject or group of subjects or a risk of exposure of a subject or group of subjects to a coronavirus or immunological relative thereof.

The method of the present invention allows for epidemiological study of outbreaks of infections caused by a coronavirus or an immunological relative thereof. Such studies provide valuable information which advance multiple facets of research. For example, epidemiological studies aid in the identification of the index case of an infection. Such information enables the identification of a defined location from which the source virus responsible for a viral outbreak originated.

Additionally, the method of the present invention permits for the rapid identification and isolation of subjects who are infected with a coronavirus or immunological relative thereof. Such information aids in identifying subjects which require medical treatment as well as defining a location which requires quarantining. Further, the method of the present invention allows for the monitoring of an infected patient to determine alterations in their viral loads. Viral load testing may be monitored to determine the efficacy of existing anti-viral treatments or aid in the development and validation of newly developed anti-viral compounds.

Further, the method of the present invention provide a means for identifying subjects

who are infected with a coronavirus, allowing for the rapid isolation of sero-positive people from the general population. Such tools allow for the rapid containment and eradication of a coronavirus or immunological relative thereof.

The present invention is further described by the following non-limiting examples.

EXAMPLE 1

SARS virus antigen

The virus (SARS CoV 2003VA2774) used in this study was isolated from a patient diagnosed with SARS the sequence of which had been previously determined Ruan *et al., Lancet 361*: 1779-1785, 2003). The virus was propagated in Vero E6 cells in medium 199 (Sigma, St Louis, USA) supplemented with 5% fetal calf serum (Biological Industries, Israel) and cultured at 37°C in 5% CO_2. The clarified culture supernatants were aliquoted into cryovials and stored at -80°C, and the virus was used in a plaque assay in Vero E6 cells in a 24 well plate format. The virus was inactivated at 60°C for an hour which was confirmed by plaque assay. Viral aliquots were stored at -20°C until use.

EXAMPLE 2

SARS positive and negative sera

Serum samples from 14 potential SARS cases (diagnosed using WHO criteria) were used in this study. Five of these were single convalescent serum samples while the other ten were paired sera collected at least two weeks apart with the first serum sample collected in the acute phase of illness. One hundred and sixty-seven SARS negative serum samples were used as controls. The control samples included serum collected from: (1) 20 laboratory staff prior to the onset of the SARS outbreak in Singapore; (2) 42 from volunteers who had a history of dengue fever; (3) 10 patients presenting with autoimmune conditions such as systemic lupus erythematosus; and (4) 95 from healthcare workers who were negative for SARS CoV neutralizing antibodies.

All serum samples were coded for subsequent blind analysis. The sera were inactivated at 56°C for half an hour before use. Assay conditions were optimized using a checkerboard titration.

EXAMPLE 3

DB-EIA protocol

The dot-blot EIA was performed onto nitrocellulose membrane (Schleincher & Schuell BA-S 83, 0.45μm, Keene, NH). The active side of the nitrocellulose (NC) membrane was marked using a pencil and 3 μl of SARS CoV suspension ($1x10^7$pfu/ml) (range from $1x10^6$ onwards) was dotted onto the active side of the NC membrane and then dried at room temperature. Each dot on the NC membrane was cut into piece (7mm x 7mm) and placed into each well of a 24- or 48-well plastic plate in such way that the labeled side was facing up. The NC dots were then blocked at 37°C for 30 minutes using blocking buffer containing 5% skim milk (w/v) (range from 1% to 10%) and 0.1% (range from 0.01% to 0.5%) Tween 20 (Duchefa, The Netherlands). After incubation, the blocking buffer was aspirated from the wells washed 3 times with washing buffer [1x PBS with 0.05% (range from 0.01% to 0.1%) Tween 20] with a short incubation of one minute between each wash. Washing was performed using gentle agitation. The membranes were then used for anti-SARS IgG assay. The antigen dotted membranes were incubated at 37°C for 15 minutes with serum sample diluted 1:100 in diluent buffer [2% (1%-3%) skim milk with 0.1% (0.05 to 0.2%) Tween 20] in duplicates. A strong, weak and negative serum controls were kept in the first column of the plate. After incubation, Ag-dotted membranes were washed four times with washing buffer. After a final wash, 150μl of rabbit anti-human IgG-HRP (1:1000 in diluent buffer) was added to each well and incubated at 37°C for 15 minutes. The strips were then washed for 5 times using washing buffer. After final wash, 150 μl of substrate solution (DAB, Code no-K3466, DakoCytomation, Denmark) was added to the Ag-dotted membranes. The presence of anti-SARS IgG was indicated with the development of well-defined circle of brownish stain within 3 minutes on NC membrane. The strips were washed 2 times with water or deionized water, dried at room temperature and results were

interpreted visually. The intensity of the stain/color developed in the dot is roughly proportional to the amount of anti-SARS IgG in the serum samples.

A temperature of 37°C was found to reduce non-specific reactions and thus produce few false positive results.

For the titration level of anti-SARS IgG, a range of dilutions of serum were made starting from 1:100 to 1:6400 and were performed in duplicates in 48 well plate format.

EXAMPLE 4

Immunofluorescence Assay (IFA)

Spot slides for immunofluorescence assay were prepared using SARS CoV infected and non-infected Vero E6 cells plated on to teflon coated slides (Erie Scientific Co, USA). SARS CoV infected cells were harvested when classified as being at stage 3+ cytopathic effects. Four microliters of cell suspension was spotted onto Teflon coated slides and fixed with 80% cold acetone for 10 minutes. Spots were also prepared using non-infected Vero E6 cells. These cell coated slides were washed three times with PBS and stored at –20°C until needed. Two microliters of test serum diluted in PBS (1:25 to 1:400) was then added in spots (both in virus infected and cell control) and incubated at 37°C for one hour. The slides were rinsed twenty times followed by soaking twice in PBS for five minutes each time. Mouse anti-human IgG antibody conjugated to FITC (Zymed, San Francisco, USA) was diluted 1:30 in Evans blue (Sigma, St Louis, USA) and 2 µl of diluted mouse anti-human IgG antibody was added to each spot and again incubated at 37°C for an hour. After washing as described above, the slides were dried and examined under a fluorescence microscope (Olympus, Japan). Results were visualized and digitally photographed.

EXAMPLE 5

Enzyme-Linked Immunosorbent Assay (ELISA)

Maxisorp F96 microplates (NUNC, USA) were coated overnight at 4°C with 50µl of SARS CoV suspension (10^6pfu/ml) in coating buffer (Fluka, Biochemica, Germany). After washing three times with washing buffer (1x PBS with 0.05% Tween 20), the unbound reactive sites were blocked with blocking (2% (w/v) skim milk with 0.1% Tween) for half an hour at 37°C. Fifty microliters of serial dilutions of serum which had been diluted in diluent buffer (2% skim milk with 0.1% Tween 20) were added into well in duplicates (1:12.5 to 1600) and plates were incubated at 37°C for an hour. After incubation, plates were washed four times with washing buffer, 50µl of rabbit anti-human IgG labeled with horseradish peroxidase diluted 1:4000 (v/v) in diluents buffer was added and the plates incubated at 37°C for an hour. Plates were washed 5 times with washing buffer followed by the addition of 50µl of substrate (OPD + H_2O_2, Sigma, USA). The reaction was stopped with 50µl of 0.1M sulfuric acid. After 15 minutes at room temperature the absorption was measured at 492 nm using ELISA reader (Anthos, 2001) and expressed as OD values. A positive reaction is indicated as the double OD values of the negative control.

EXAMPLE 6

Comparison of DB-EIA, ELISA and IFA

DB-EIA was developed using SARS CoV serum recovered from 4 subjects which were confirmed positive for SARS and 10 SARS negative sera collected a year ago, of which 4 tested positive for dengue IgG. Sera were tested in dilution ranges of 1:12.5 to 1:400. Results demonstrated that the sera from all 4 patients which had been previously confirmed as being positive for SARS CoV were positive for SARS CoV using DB-EIA, while all 10 SARS CoV negative samples did not show any reaction (see Figure 1).

The 10 SARS confirmed sera were further titrated in ranges of dilutions of 1:100 to 1:6400. Again, all 4 samples tested positive at the lowest dilution, i.e. 1:6400 (see Figure 3).

The same serum samples were also examined using ELISA and IFA. Results showed a high level of background in both the assays and a number of SARS CoV negative sera showed a high level of cross-reactivity using IFA (see Table 1). In ELISA and IFA, both assays showed a low level of sensitivity and could not detect anti-SARS IgG below the 1:800 and 1:400 dilutions, respectively (see Figure 2 and Figure 3).

EXAMPLE 7

Diagnosis using the DB-EIA protocol

Ten paired samples from SARS confirmed patients were examined using the DB-EIA protocol described above. Serum samples were collected from SARS suspected patients at the time of admission in hospital and at various time points between day 15-21 post onset of fever. Results obtained showed that all 10 patients' sera demonstrated high levels of specific reactivity (see Figure 3).

Additionally, the sensitivity and specificity of DB-EIA were confirmed using 14 SARS confirmed convalescent sera and 167 SARS negative sera. All 14 SARS convalescent sera showed a clear positive reaction with DB-EIA, thus detecting 100% of the samples tested. All 167 SARS CoV negative samples were negative using DB-EIA, demonstrating 100% accuracy in the diagnostic capabilities of the assay (Table-1). A Chi-squared analysis with Yates continuity correction showed that this is a highly significant finding (χ^2=166.70; p=0.0005).

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TABLE 1

χ^2 analysis of the difference in frequencies of SARS confirmed positive and negative sera tested.

DB-EIA

	Positive	Negative	
Positive	14	0	14
Negative	0	167	167
	14	167	181

SARS

$\chi^2 = 166.70$; odds ratio = infinity

CLAIMS

1. A method for detecting exposure of an animal to a coronavirus or immunological relative thereof, comprising the steps of:

(a) contacting a biological sample from said animal with a component derived from said coronavirus or an immunological relative thereof for a time and under conditions sufficient to facilitate the formation of a complex between said component and a subject-derived binding partner of the component contained within said biological sample; and

(b) determining the presence of said complex, wherein the presence of said complex is indicative of exposure of said subject to a coronavirus or immunological relative thereof.

2. The method of claim 1, wherein the component is a polypeptide, glycoprotein, lipid, carbohydrate, DNA, RNA or RNAi.

3. The method of claim 1, wherein the component is a polypeptide.

4. The method of claim 3, wherein the polypeptide is selected from the group consisting of small envelope protein Type II, membrane glycoprotein M, Orf3, Orf4, Orf7, Orf8 Type I, Orf9 Type II, Orf10, Orf11, spike glycoprotein Type I, replicase IA/IB and nucleocapsid protein.

5. The method of any one of claims 1 to 4, wherein the subject-derived binding partner of said component is an antibody.

6. The method of any one of claims 1 to 5, wherein detection is during acute infection.

7. The method of any one of claims 1 to 5, wherein detection is post-infection.

8. The method of any one of claims 1 to 7, wherein said coronavirus causes symptoms associated with a lower respiratory tract infection.

9. The method of any one of claims 1 to 8, wherein said coronavirus causes SARS or symptoms associated with SARS.

10. The method of any one of claims 1 to 9, wherein the biological sample is selected from the group consisting of blood, PBCMs, stem cells, monocytes, T cells, B cells, macrophages, dendritic cells, natural killer cells, amniotic fluid, placenta, chorionic villus, cord blood, hair, finger nails, bones, cartilage, skin, saliva, serum, plasma, teeth, urine and tissue.

11. The method of any one of claims 1 to 10, wherein said biological sample is placed onto a membrane prior to being contacted with said component.

12. The method of any one of claims 1 to 10, wherein said component is placed onto a membrane prior to being contacted with the biological sample.

13. The method of any one of claims 11 and 12, wherein said membrane is a nitrocellulose membrane.

14. A method for detecting exposure of an animal to a coronavirus or immunological relative thereof, comprising the steps of:

(a) contacting a biological sample from said animal with a component derived from said coronavirus or an immunological relative thereof for a time and under conditions sufficient to facilitate the formation of a complex between said component and a subject-derived immunointeractive molecule of the component contained within said biological sample; and

(b) determining the presence of said complex, wherein the presence of said complex is indicative of exposure of said subject to a coronavirus or immunological relative thereof.

15. The method of claim 14, wherein the component is a polypeptide, glycoprotein, lipid, carbohydrate, DNA, RNA or RNAi.

16. The method of claim 14, wherein the component is a polypeptide.

17. The method of claim 16, wherein the polypeptide is selected from the group consisting of small envelope protein Type II, membrane glycoprotein M, Orf3, Orf4, Orf7, Orf8 Type I, Orf9 Type II, Orf10, Orf11, spike glycoprotein Type I, replicase IA/IB and nucleocapsid protein.

18. The method of any one of claims 14 to 17, wherein the subject-derived binding partner of said component is an antibody.

19. The method of any one of claims 14 to 18, wherein detection is during acute infection.

20. The method of any one of claims 14 to 18, wherein detection is post-infection.

21. The method of any one of claims 14 to 20, wherein said coronavirus causes symptoms associated with a lower respiratory tract infection.

22. The method of any one of claims 14 to 21, wherein said coronavirus causes SARS or symptoms associated with SARS.

23. The method of any one of claims 14 to 22, wherein the biological sample is selected from the group consisting of blood, PBCMs, stem cells, monocytes, T cells, B cells, macrophages, dendritic cells, natural killer cells, amniotic fluid, placenta, chorionic villus, cord blood, hair, finger nails, bones, cartilage, skin, saliva, serum, plasma, teeth, urine and tissue.

24. The method of any one of claims 14 to 23, wherein said biological sample is placed onto a membrane prior to being contacted with said component.

25. The method of any one of claims 14 to 23, wherein said component is placed onto a membrane prior to being contacted with the biological sample.

26. The method of any one of claims 24 and 25, wherein said membrane is a nitrocellulose membrane.

27. A method for assessing the relative risk of one or more subjects being exposed to a coronavirus or immunological relative thereof within a defined location, comprising:

(a) obtaining biological samples from a representative population within the defined location; and

(b) assessing evidence of exposure of individual members of a sample population to a coronavirus or immunological relative thereof by the steps of:

(i) contacting a biological sample from the subject with a component derived from the coronavirus or immunological relative thereof for a time and under conditions sufficient to facilitate the formation of a complex between the component and a subject-derived binding partner of the component contained within the biological sample; and

(ii) determining the presence of the complex, wherein the presence of the complex is indicative of exposure of the subject to a coronavirus or immunological relative thereof,

and thereby assessing the relative risk of exposure within the defined location.

28. A method for assessing the relative risk of one or more subjects being exposed to a coronavirus or immunological relative thereof within a defined location, comprising:

(a) obtaining biological samples from a representative population within defined location; and

(b) assessing evidence of exposure of individual members of a sample population to a coronavirus or immunological relative thereof by the steps of:

(i) contacting a biological sample from the subject with a component derived from the coronavirus or immunological relative thereof for a time and under conditions sufficient to facilitate the formation of a complex between the component and subject derived immunointeractive molecule contained within the biological sample; and

(ii) determining the presence of the complex, wherein the presence of the complex is indicative of exposure of the subject to a coronavirus or immunological relative thereof,

and thereby assessing the relative risk of exposure within the defined location.