ANTIBODY RESPONSE TO SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS: A CANADIAN PERSPECTIVE

By
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A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
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Winnipeg, Manitoba
Canada

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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Of

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aa	amino acids
AcMNPV	*Autographa californica* multiple nucleopolyhedrovirus
Amp	ampicillin
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
CoV(s)	coronavirus(es)
CPE	cytopathic effect
DMEM	Dulbecco's modified Eagle's medium
dNTP	deoxynucleoside triphosphate
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
His	histidine
HRP	horse radish peroxidase
Ig	immunoglobulin
IFA	immunofluorescence assay
kDa	kilodaltons
LB medium	Luria-Bertani medium
MEM	minimum essential medium
M.O.I	multiplicity of infection
mRNA	messenger ribonucleic acid
H_2SO_4	sulfuric acid
NaCl	sodium chloride
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFU	plaque forming unit
RNA	ribonucleic acid
RNase	ribonuclease
RT-PCR	reverse-transcription polymerase chain reaction
SARS	Severe Acute Respiratory Syndrome
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sf21	*Spodoptera frugiperda* 21 cells
SFM	serum free medium
$TCID_{50}$	tissue culture infectious dose
TMB	teteramethylbenzidine
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranosid

ABSTRACT

SARS-CoV is the etiological agent of the newly emerged human disease, SARS, and responsible for a global outbreak of atypical pneumonia. As a novel virus, the immunology and characteristics of SARS-CoV infection are not fully understood. To gain insight into the immune response to SARS-CoV infection, the presence and profile of antibodies against SARS-CoV nucleocapsid (N), spike (S), and matrix (M) proteins were evaluated in Canadian probable and suspect cases. From 132 SARS-CoV infected patients, 194 serum samples collected from days 2 to 256 after the onset of illness were analyzed using recombinant SARS-CoV protein-based ELISA and Western blotting. Detection of protein specific antibodies by ELISA revealed that the SARS-CoV N protein elicits the earliest and strongest immune response throughout the course of the disease (97.9%), followed by S (73.7%), E (30.9%) and M (28.3%) proteins. Further profiling of the IgG, IgA, and IgM antibodies against the SARS-CoV N protein demonstrated an IgG-dominated antibody response with respect to the titer and positive rate of detection. Similar results were obtained by Western blotting. Removal of the C-terminal region of SARS-CoV N protein abolished all reactivity with SARS positive patient sera. In contrast, truncating the N-terminal region did not have an adverse effect on detection rates. Collectively, this data suggests that the immunodominant region of SARS-CoV N protein lies within the C-terminus.

The present study also assesses the specificity between antibodies to human coronaviruses SARS, OC43, and 229E in SARS positive, negative and non-SARS sera. Based on recombinant SARS-CoV nucleocapsid protein-based ELISA, immunoblot

analysis, and competition assays, antibodies against SARS-CoV and HCoV-229E cross-react more frequently than antibodies against SARS-CoV and HCoV-OC43. Removal of the N-terminus of SARS-CoV N, containing a highly conserved motif between all HCoVs, reduced the cross-reactivity between anti-HCoV nucleocapsid protein antibodies.

1 INTRODUCTION

1.1 History

In November 2002, an outbreak of atypical pneumonia, designated severe acute respiratory syndrome (SARS), first emerged in Guangdong Province, China (Figure 1). Within months of its appearance, the disease was recognized as a global threat and subsequently spread to other Asian countries, North America and Europe. During the peak of the worldwide outbreak, more than 200 new cases were being reported each day. Through collaboration of the scientific community, the causative agent of SARS was rapidly identified as a novel coronavirus, designated severe acute respiratory syndrome coronavirus (SARS-CoV), just five months after its initial appearance (Drosten *et al.*, 2003; Ksiazek *et al.*, 2003; Peiris *et al.*, 2003). In July 2003, the World Health Organization (WHO) announced the last known chain of human-to-human transmission had been broken and that the global outbreak was over. However, despite tremendous concerted efforts, SARS had spread to over 32 countries and regions worldwide, infecting more than 8400 people and causing over 800 deaths (WHO, 2003) (Figure 2).

With respect to Canada, the worldwide outbreak of SARS reached Toronto in February 2003, after a traveler that had been exposed in Hong Kong returned home (Poutanen *et al.*, 2003; Tang *et al.*, 2004). Subsequent transmission to family and other community members resulted in an outbreak among 257 people and 27 deaths, the majority of whom were health care workers, patients, or visitors (Hui *et al.*, 2004). By mid-May, the epidemic was thought to be over after the initial outbreak had primarily

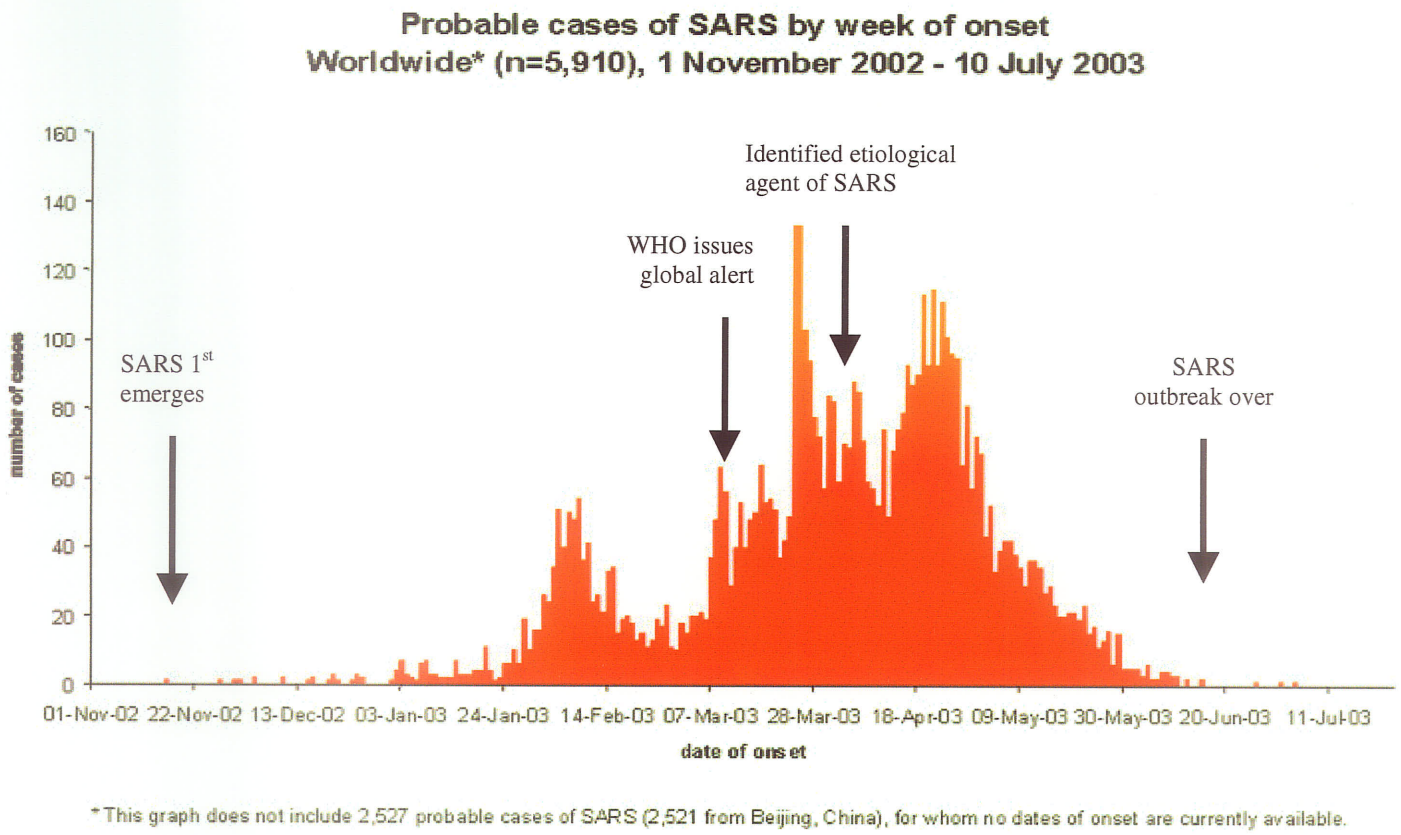

Figure 1. Timeline of probable cases of severe acute respiratory syndrome (SARS) from November 1, 2002 to July 10, 2003, highlighting key events. Adapted from WHO epidemic curves (http://www.who.int/csr/sars/epicurve/epiindex/en/index1.html)

Figure 2. Probable cases of severe acute respiratory syndrome (SARS) reported worldwide between 1 November 2002 to 31 July 2003 (WHO). [a]Includes France, Germany, Ireland, Italy, Romania, Spain, Sweden, Switzerland, and United Kingdom. [b]Includes Macao. ●, Date of onset of first probable case; □, Date of onset of last probable case (Christian *et al.*, 2004)

been contained. However, a second wave of SARS cases emerged, with an additional 119 probable and suspect cases, and 17 deaths (Tang *et al.,* 2004) (Figure 3). As a result, Canada faced the second largest SARS outbreak, aside from Asia, with at least 438 probable or suspected cases and 43 deaths, mostly in the Toronto area (Health Canada, 2003).

1.2 Clinical Features and Case Management

Coronaviruses can cause severe enteric and respiratory diseases in many animals such as infectious bronchitis virus (IBV) in chickens, feline infectious peritonitis virus (FIPV) in cats, mouse hepatitis virus (MHV) in mice, and transmissible gastroenteritis CoV (TGEV) in pigs (Lai and Holmes, 2001). In contrast, human CoVs have been associated only with ~30% of mild upper respiratory tract illnesses, until now. The novel SARS-CoV appears to be the first human coronavirus responsible for severe disease in humans.

Similar to most other CoVs, SARS infects the epithelial cells of the respiratory and/or enteric tract. The virus has a mean incubation period of 6.4 days, which can vary from 2 to 16 days (Knudsen, *et al.*, 2003). Following infection, patients generally follow a typical 3-phase clinical course (Pieris *et al.*, 2003; Rainer *et al.*, 2004). In the initial phase of illness, the clinical symptoms of SARS are rather non-specific and may mimic influenza or atypical pneumonia. This phase is associated with increasing viral load and clinically characterized by fever, chills/rigor, myalgia, malaise, dry cough, headache, and dyspnoea that lasts a few days and then resolves. Progression into phase 2 begins around day 8 after fever onset and is marked by immune-mediated lung injury accompanied by

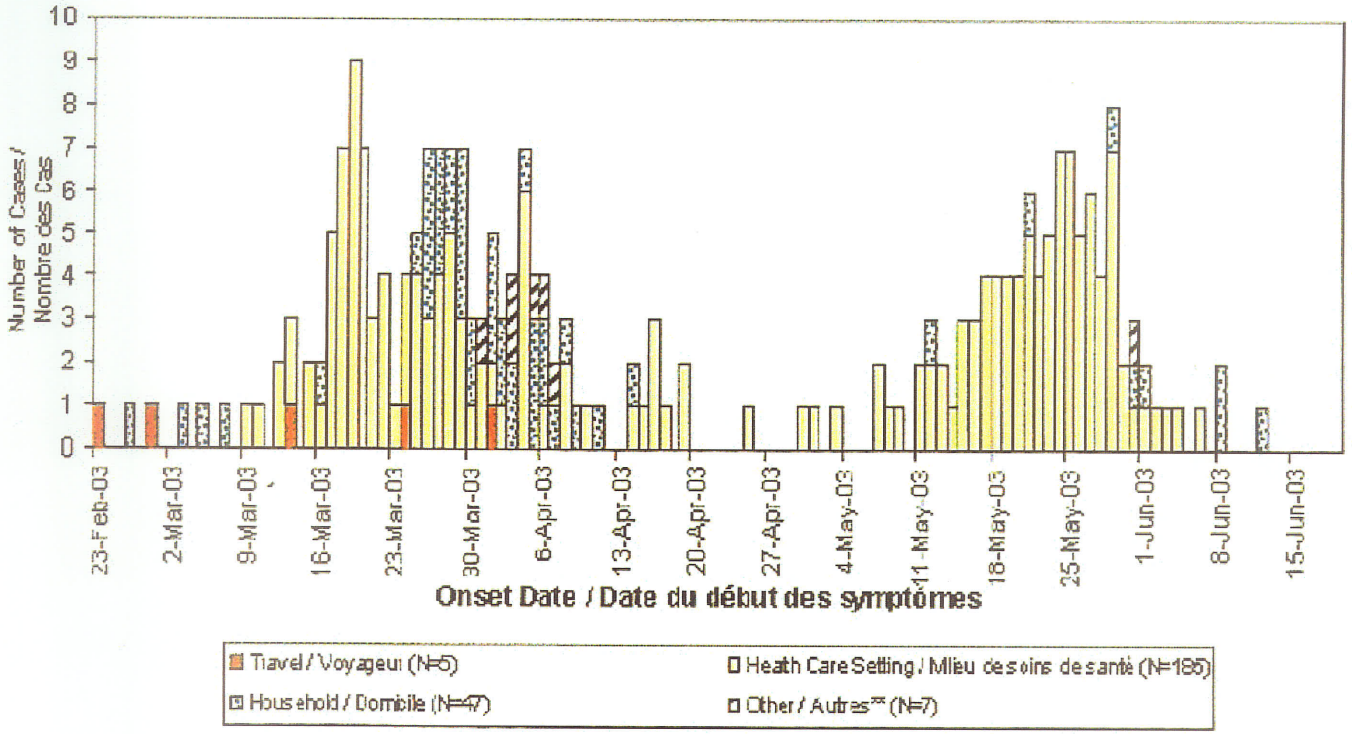

Figure 3. The distribution of SARS cases in Canada from February 23 to June 12, 2003 (http://www.who.int/csr/sars/epicurve/epiindex/en/index1.html)

falls in viral load. Clinically, this phase is distinguished by reoccurrence of fever, and progression of pneumonia. Although the majority of patients will improve and enter a third phase of rehabilitation, 20% of patients may suffer from severe lung injury characterized by acute respiratory distress syndrome that requires invasive ventilatory support (Lee *et al.,* 2003; Peiris, *et al.,* 2003). Death from progressive respiratory failure occurs in about 10% of cases (Lee *et al.*, 2003). The most important risk factor for the development of severe disease and death is increasing age, as patients over the age of 60 years have a case fatality rate of 45% (Booth *et al.*, 2003; Peiris *et al.*, 2003).

Due to the limited understanding of the pathogenesis and clinical course of this newly emerged disease, there are currently no antiviral drugs, immunotherapeutic agents, or vaccines available against the virus. The existing available strategy for the control of SARS is isolation and quarantine.

1.3 SARS-CoV Diagnosis

Initially, strategies for the detection of the novel agent included electron microscopy, virus isolation, viral sequence identification and serological testing (Drosten *et al.*, 2003; Ksiazek *et al.*, 2003; Marra *et al.*, 2003; Peiris *et al.*, 2003; Rota *et al.*, 2003). Among these, fall two approaches that are currently used for diagnosing SARS-CoV infection in humans. The first method is based on molecular detection of SARS-CoV RNA in clinical specimens. Although this technique is the most sensitive in detecting early SARS-CoV infection, the positive predictive rate for probable SARS is only 32% (Peiris *et al.*, 2003). Thus, the second and most widely used method for diagnosing SARS-CoV infection is by detecting antibodies against the virus, either by

immunofluorescence assay (IFA), neutralization assay, enzyme-linked immunosorbent assay (ELISA), or Western blot (WB) assay. Often these tests are based on antibodies reacting with cell culture extract. Due to the severity of SARS disease and the contagious nature of the causal agent, a biosafety level 3 laboratory is required to cultivate SARS-CoV. Such facilities are not available in most clinical microbiology laboratories. To overcome this problem, Western blot and ELISA-based antibody detection test using recombinant antigens may be employed. Of these two methods, ELISA assay would be more practical as it can screen a greater number of samples in less time, which is critical during an outbreak. Therefore, not only do recombinant protein-based ELISAs eliminate the need to cultivate the virus, numerous studies have shown that a recombinant SARS protein-based ELISA could serve as a simple and sensitive test for diagnosing SARS-CoV infection (Guan *et al.*, 2004; Woo *et al.*, 2004). Another consideration to be made is the specificity of the test. Thus, SARS-CoV proteins must be evaluated for possible serological cross-reaction amongst human coronaviruses.

1.4 Transmission

Unlike other respiratory viruses, which are most transmissible in the early stages of disease, SARS-CoV appears to be most infectious during the second week of a patient's illness. The transmission of SARS occurs primarily through respiratory droplets and generally requires close contact with a symptomatic patient (Scales *et al.*, 2003; Loeb *et al.*, 2004). However, evidence for airborne spread of SARS-CoV remains inconclusive (WHO). Virus particles have been shown to be fairly stable, as they remain infectious for up to 48 hours after drying on plastic surfaces and possibly up to 4 days in feces (WHO,

2004; Booth *et al., 2005*). This raises the possibility that fomites, and fecal-oral transmission may play a role in the spread of SARS.

1.5 Phylogeny

SARS-CoV belongs to a diverse family of viruses that are classified within the order Nidovirales, family *Coronaviridae,* genus *Coronavirus.* Until recently, there were three antigenic groups of coronaviruses: groups 1 and 2 contain mammalian viruses, whereas group 3 contains only avian viruses. The two previously identified human CoVs are found in both group 1 (229E) and group 2 (OC43). Phylogenetic analyses and sequence comparisons show that SARS-CoV is not closely related to any of the known coronaviruses, sharing only 25-30% identity with other members of the Coronavirus family (Figure 4) (Ksiazek *et al.*, 2003; Marra *et al.*, 2003; Rota *et al.*, 2003). Thus, SARS-CoV forms a distinct group within the genus *Coronavirus.* Based on the replicase gene, SARS' closest relatives are the murine, bovine, porcine, and human CoVs of group II and avian CoV of group I (Snijder *et al.*, 2003; Zhu & Chen, 2004).

The origin and evolutionary history of SARS-CoV has been the subject of much uncertainty and debate. Like most emerging infectious diseases, SARS is believed to be a viral zoonotic disease, as more than one-third of early cases of SARS in Guangdong were animal or food handlers (He *et al.*, 2003). Seroprevalence studies reinforced this zoonotic link as 13% of asymptomatic animal handlers at markets in Guangdong Province had antibodies against SARS-CoV (CDC, 2003). In support, a SARS-CoV-like virus, which shares 99.8% sequence identity to human SARS-CoV, has recently been isolated from animals, such as civet cats and raccoon dogs in live animal markets (Guan *et al.*, 2003).

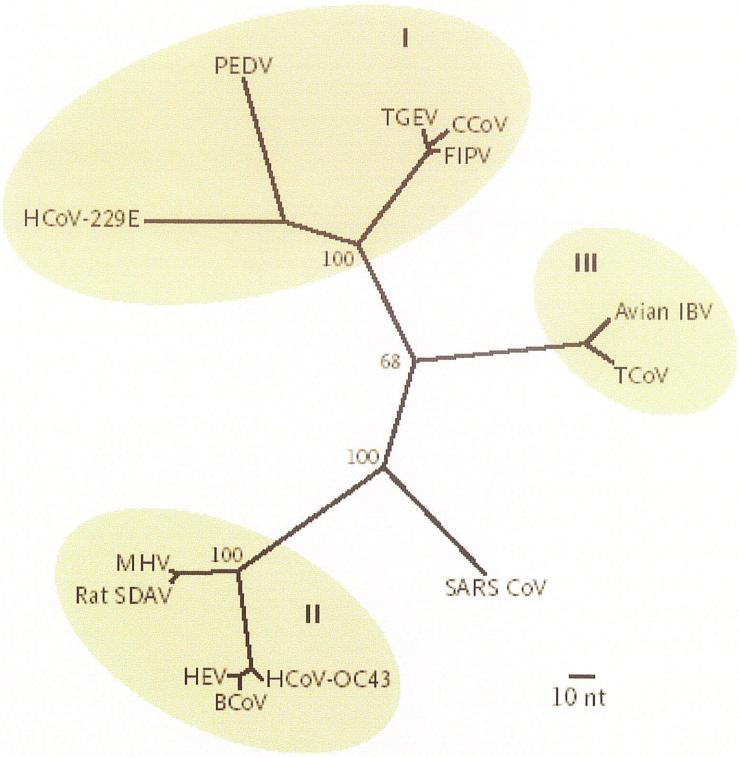

Figure 4. Phylogenetic analysis of the polymerase gene, comparing SARS-CoV with known coronaviruses. The polymerase gene sequences were aligned with those of known coronaviruses and a maximum-parsimony tree was constructed. The three major coronavirus antigenic groups (I, II, III) are represented by human coronavirus 229E (HCoV-229E), canine coronavirus (CCoV), feline infectious peritonitis virus (FIPV), porcine transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhea virus (PEDV), human coronavirus OC43 (HCoV-OC43), bovine coronavirus (BCoV), porcine hemagglutinating encephalomyeltitis virus (HEV), rat sialodacryoadenitis virus (SDAV), mouse hepatitis virus (MHV), turkey coronavirus (TCoV), and avian infectious bronchitis virus (IBV). The main internal branches of the phylogram display the bootstrap values (from 100 replicates) obtained from a 50 % majority rule consensus tree. Branch lengths are proportionate to nucleotide differences (Ksiazek *et al.*, 2003)

Furthermore, recent serological studies suggest that transmission of animal SARS-like CoV to humans may have been occurring undetected for at least 2 years prior to the recent SARS outbreak (Zheng *et al.,* 2004). Over time, adaptation of the SARS-like CoV appears to have created the more virulent SARS-CoV capable of human-to-human transmission.

Viral zoonotic diseases often originate from the genetic exchange between viruses with different host specificities. It was originally believed that SARS-CoV did not arise by mutation or recombination between established coronaviruses, due to its low sequence identity to known CoVs (Holmes, 2003; Marra *et al.*, 2003; Rota *et al.*, 2003). However, recent studies indicate that SARS-CoV is in fact a mosaic of mammalian and avian-like viruses that may have arisen from a combination of host jumping and recombinational events. For instance, evolutionary analyses of CoV DNA sequences reveal that the replicase protein has a mammalian-like origin, the M and N protein have an avian-like origin and the S protein has a mammalian-avian mosaic origin, as illustrated in Figure 5 (Stanhope *et al.*, 2004; Stavrindes & Guttman, 2004). Adding further support, Zhang *et al.* (2004) identified seven putative recombination regions that encompass the replicase 1A, replicase 1B, and the spike glycoprotein. Since the S protein is responsible for host specificity, the recombination event that occurred within the S gene may have been the critical step in the switch to a human host and the subsequent emergence of this new pathogen.

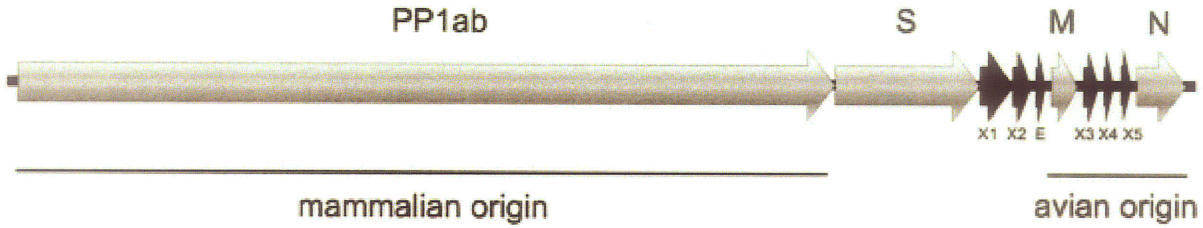

Figure 5. Organization of the SARS-CoV genome displaying the possible past recombination event between mammalian-like and avian-like parent viruses. The analyses supports a mammalian-like origin for the replicase protein, an avian-like origin for the matrix and nucleocapsid proteins and a mammalian-avian mosaic origin for the spike protein (Stavrinides et al., 2004)

1.6 Structure and Genome

Coronaviruses have a unique morphologic appearance characterized by large club-shaped surface projections, extending out of a host cell-derived envelope that encases a coiled helical nucleocapsid structure (Figure 6a). The virions are approximately 75 to 160 nm in diameter, and appear on electron micrographs as pleomorphic particles, with a distinctive 'crown-like' appearance, from which the family was given its name (Figure 6b). The genome consists of a single linear molecule of plus-sense RNA, which ranges in length from 27 to 33 kb, making it the largest of all RNA virus genomes. The SARS-CoV genome is 29,751 nucleotides in length and contains 14 potential open reading frames (ORFs) (Marra *et al.*, 2003). The genomic organization is similar to that of other coronaviruses with a gene order [5'- replicase (rep), spike (S), envelope (E), membrane (M), and nucleocapsid (N) -3'] and short untranslated regions at both termini (Marra *et al.*, 2003) (Figure 6c). The SARS-CoV replicase gene (rep), which comprises approximately two-thirds of the genome, contains two ORFs, 1A and 1B, which encode polyproteins of the replicase complex. Downstream of the rep gene are ORFs encoding the four main structural proteins: spike glycoprotein (S), envelope glycoprotein (E), membrane (M), and nucleocapsid (N) proteins. Together they function during host cell entry, virion morphogenesis, and release (Chan *et al*, 2003). In addition, there are several other ORFs that have been identified, which may encode additional proteins; however, their functions are currently unknown.

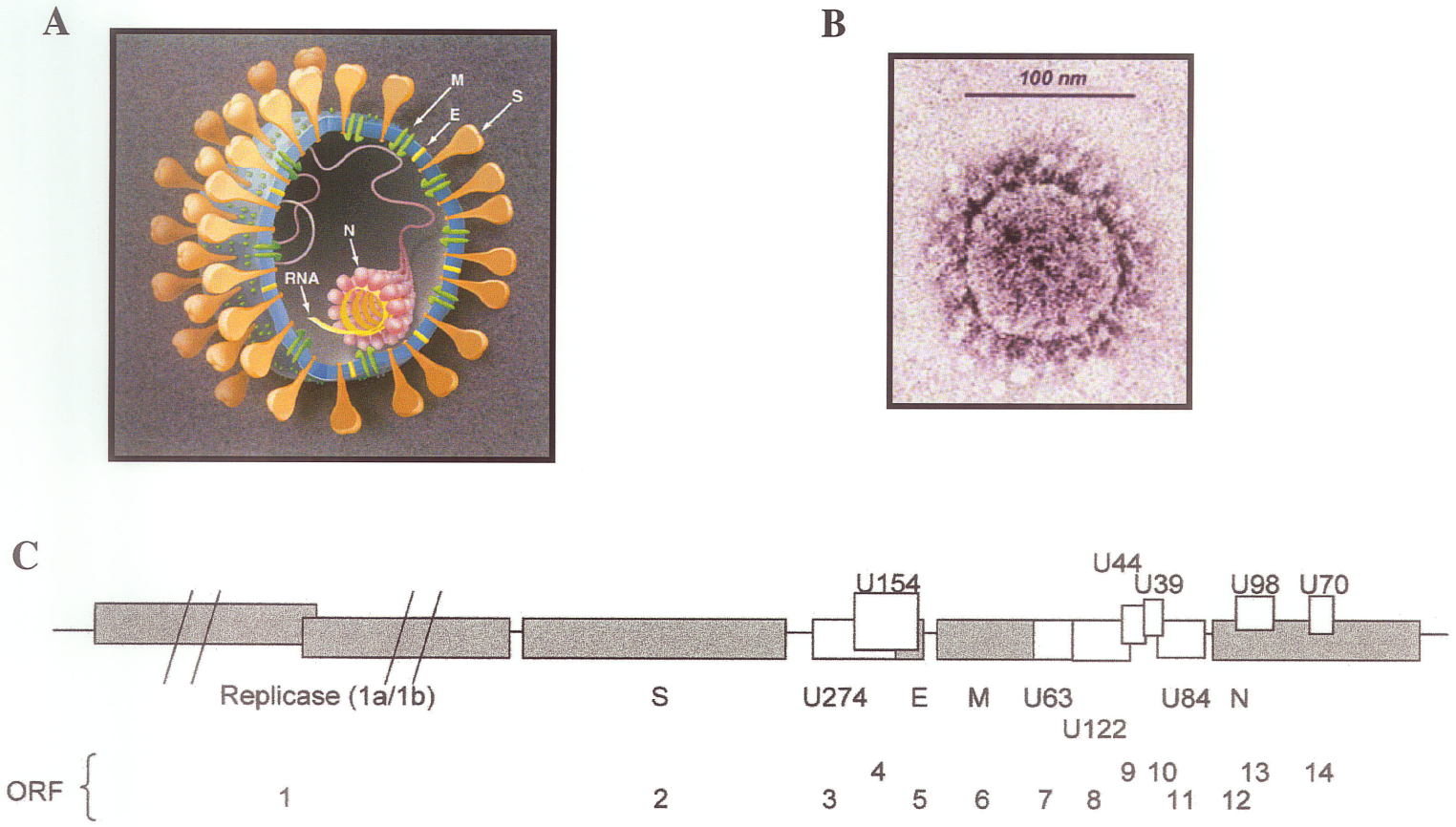

Figure 6. Structural organization of SARS-CoV. A) Model of the SARS-CoV structure showing the organization of the structural proteins and helical nucleocapsid (Holmes *et al.*, 2003). B) Electron micrograph of the ultrastructural characteristics of SARS-CoV (Ksiazek *et al.*, 2003). C) Genetic organization of SARS-CoV. ORFs corresponding to structural proteins S, M,N, E and unique proteins (UX). X denotes the number of amino acids encoded by the respective ORF (Tan *et al.*, 2004)

1.6.1 Envelope (E) protein

SARS-CoV envelope (E) gene encodes a small envelope protein of 76 amino acids that is present in minute amounts in the viral envelope. As a membrane protein, the major biological function of the E protein is to participate in virus budding and morphogenesis (Shen *et al.*, 2003).

1.6.2 Membrane (M) protein

The membrane (M) gene encodes a protein of 221 amino acids and is a major component of the viral envelope involved in viral assembly and budding. In the cases of other known coronaviruses, it is the most abundant structural protein and contributes to generating the host immune response against many coronaviruses, including TGEV, IBV, pig-respiratory CoV and MHV (Lu *et al.*, 2004). Furthermore, M-specific antibodies of known coronaviruses both neutralize and mediate the complement-dependent lysis of infected cells (Woods *et al.*, 1987; Risco *et al.*, 1995). More recently, studies have shown that the M protein can induce a strong, SARS-CoV specific neutralizing antibody response (Pang *et al.*, 2004). As well, recombinant M protein reacts with SARS sera, indicating the induction of an immunological response by the M protein. (Guan *et al.*, 2004; Han *et al.*, 2004).

1.6.3 Spike (S) glycoprotein

The spike (S) protein is a large membrane glycoprotein and forms 180-190 kDa peplomers that produce the characteristic "club-shaped" or "crown-like" viral projections attributed to all CoVs. As the major surface antigen of the virus, the S protein plays a critical role in the biology and pathogenesis of coronaviruses (Lai *et al.*, 2001; Ren *et al.*,

2003). In the initial stages of infection, the spike protein is responsible for binding to cellular receptors and inducing membrane fusion, both of which are critical for virus entry into target cells (Gallagher *et al.*, 2001). As such, the S protein is the major neutralizing antigen of the known CoVs (Homberger *et al.*; 1994Callebaut *et al.*, 1996; Gomez *et al.*, 1998). In addition, recent studies have identified the spike protein as a key target of the cellular immune response (Wang *et al.*, 2003; Lu *et al.*, 2004; Ren *et al.*, 2003). As a result, the spike glycoprotein is of major interest as a target of antiviral drug development as well as for developing a vaccine against SARS-CoV.

1.6.4 Nucleocapsid (N) protein

The nucleocapsid (N) gene of SARS-CoV encodes a protein of 422 amino acids that binds to the viral genome to form the nucleocapsid. The N protein is abundantly produced during viral infection and relatively conserved among SARS-CoV strains (Li *et al.*, 2003; Ruan *et al.*, 2003; Tong, *et al.*, 2004; Wang, *et al.*, 2004). Based on available information of other coronaviruses, the nucleocapsid protein has multiple functions that include: nuclear import signaling, interfering with host cell cycle processes, and participating in the replication and transcription of viral RNA (Parker *et al.*, 1990; Kuo *et al.*, 2002). Current research has found that SARS-CoV N protein is capable of self-multimerization suggesting an important role in the formation of the viral nucleocapsid core (He *et* al., 2004). Antigenic studies have shown that the N protein of CoVs possesses strong antigenicity and plays an important role in the induction of the host humoral and cellular immune responses (Bergmann *et al.*, 1993; Ignjatovic et al., 1993; Stohlman *et al.*, 1994; Rodriguez *et al.*, 1997; Casal *et al.*, 1998; Wootton, *et al.*, 2001).

Furthermore, the nucleocapsid proteins are capable of inducing protective immune responses against CoV infection (Boots *et al.*, 1992; Seo *et al.*, 1997; Wege *et al.*, 1993; Wesseling *et al.*, 1993). Recent studies have discovered that the antibodies to SARS-CoV N protein were highly detectable in SARS patients (Krokhin *et al.*, 2003; Liu *et al.*, 2004; Shi *et al.*, 2003; Wang *et* al, 2003), suggesting that this protein may serve as one of the immunodominant antigens in the diagnosis of infection.

1.7 Objectives

SARS was the first severe and readily transmissible novel disease to emerge in the 21st century. Immediately perceived as a global threat with pandemic potential, an international concerted effort was initiated to isolate and identify the etiological agent of SARS. After its rapid identification, subsequent research was focused on characterizing SARS-CoV infection to facilitate the development of various therapeutic strategies and vaccines, in addition to producing a sensitive and specific diagnostic tool. Both of which are critical in controlling the spread of future outbreaks. Despite remarkable strides, a complete understanding of SARS-CoV pathogenesis and the human immune response to infection has not yet been achieved. Furthermore, potential problems in developing a diagnostic tool, such as serological cross-reactivity amongst human coronaviruses, must still be assessed. Thus, the present study was established to analyze the presence and profile of SARS-CoV specific antibodies. In addition, it will assess the serological cross-reactivity amongst human coronaviruses and identify immunodominant domains of SARS-CoV proteins. Taken together, this data will be critical in developing a reliable diagnostic tool.

The specific objectives include:

1) To construct recombinant SARS-CoV proteins using a baculovirus expression system in order to:

   (a) Measure the human IgG antibody response to viral proteins encoded by the SARS-CoV genome to assess the changes in antibody response to each protein throughout the course of the disease.

(b) Profile the human IgG, IgA, and IgM response to SARS-CoV nucleocapsid protein to determine the titer and specific response of each antibody throughout the course of the disease.

2) To analyse recombinant SARS, OC43, and 229E CoV nucleocapsid proteins as potential antigens using a baculovirus expression system in order to:

(a) Investigate the presence of antibodies against OC43 and 229E in serum samples.

(b) Assess specificity between antibodies to human coronaviruses SARS, OC43, and 229E.

3) To express truncated human CoV nucleocapsid proteins using a baculovirus expression system in order to:

(a) Identify immunodominant region of SARS-CoV N protein.

(b) Identify region(s) responsible for cross-reaction between antibodies against human coronavirus N proteins.

The Baculovirus Expression System (BES) was chosen for this study, as it is one of the most powerful and versatile eukaryotic systems available. There are many advantages of using this system including: limitless size of the expressed protein, efficient cleavage of signal peptides, intron splicing, nuclear transport, and simultaneous expression of multiple genes. In addition, the expressed recombinant proteins contain proper folding, disulfide bond formation and oligomerization. Furthermore, this system is capable of performing several post-translational modifications (i.e. glycosylation, phosphorylation, acylation) to produce proteins similar in structure and function to their native form. This proves important for this study as the M, S, and E protein of SARS are

glycoproteins and their modification is important for their activity and effects (Han *et al.*, 2004). Lastly, a major distinguishing feature of the BES is the presence of a powerful polyhedron promoter, which can drive high-level expression of a cloned gene, resulting in large quantities of the protein of interest (Luckow, *et al.*, 1988).

1.8 Significance of the Study

As a novel virus, the immunology and characteristics of SARS-CoV infection are not fully understood. The present study will enable us to gain insight into these critical features using suspect and probable serum samples collected in Canada.

The implications of these studies are immense, as they will provide crucial information about SARS-CoV pathogenesis and the human immune response to infection. Detailed knowledge of the presence and profile of SARS-CoV specific antibodies and identification of protein domains responsible for immunodominance and cross-reactivity may lead to new concepts for diagnosis, prevention and therapeutic strategies to combat SARS-CoV infection.

2 MATERIALS AND METHODS

2.1 Cells and Viruses

Vero E6 (green monkey kidney) cells were cultured in Eagle's minimum essential medium (MEM, Invitrogen) with 10% heat inactivated fetal bovine serum (FBS), 25 mM HEPES buffer, 0.1 mM MEM Non-Essential Amino Acid solution, 1.0 mM MEM Sodium Pyruvate solution and 1% penicillin/streptomycin/glutamine. The cells were incubated in the presence of 5% CO_2 at 37°C.

Sf21 (*Spodoptera frugiperda*) cells were cultured in Grace's insect cell culture medium (Invitrogen) supplemented with 10% heat inactivated FBS and 1% penicillin/streptomycin/kanamycin. The cells were incubated at 27°C.

Escherichia coli TOP 10 chemically competent cells were purchased from Invitrogen.

SARS-CoV (Tor2 strain) was originally isolated from a bronchoalveolar lavage specimen of a fatal SARS case from Toronto, Canada (isolate Tor2; GenBank accession number NC_004718). SARS-CoV was propagated in Vero E6 with MEM containing 2% FBS, 25 mM HEPES buffer, 0.1 mM MEM Non-Essential Amino Acid solution, 1.0 mM MEM Sodium Pyruvate solution and 1% penicillin/streptomycin/glutamine. The virus was grown at 37°C, in the presence of 5% CO_2. To prepare SARS-CoV crude viral antigens, SARS-CoV (Tor2) infected Vero E6 cells were harvested 72 h post infection, and washed with cold phosphate-buffered saline. After brief centrifugation (3000 rpm, 5 min), cells were resuspended in distilled water, and an equal volume of 2X sample buffer (100mM Tris-HCL, pH7.5, 10% (v/v) β-mercaptoethanol, 0.2% (w/v) bromophenol blue,

4% (w/v) SDS, 25% (v/v) glycerol) was added. The cell lysate was γ-irradiated and heated for 10 min at 95°C. Viral growth and isolation was done in a biosafety level (BSL) 3 laboratory and all handling of infectious SARS-CoV was performed under appropriate biocontainment conditions as outlined in the Public Health Agency of Canada Laboratory Biosafety Guidelines (http://www.phac-aspc.gc.ca/publicat/lbg-ldmbl-96/lbg7_e.html).

2.2 Human Sera

A database containing information on all probable and suspected SARS sera collected in Canada, along with the corresponding collection day post onset of illness, served as the foundation for this project. An additional database consisting of data on non-SARS sera, accompanied by information on gender and age was also used for this research. The sera used for the antibody response and specificity studies are described below and summarized in Table 1.

1) Sera collected prior to the SARS outbreak, designated non-SARS sera. Serum specimens were collected from the Saskatchewan provincial laboratory from patients admitted to the hospital in 2000 and 2001. These serum samples were used as non-SARS sera controls in the study. The age of patients ranged from 16 to >60 years old, with 50% of the tested serum being female.
2) Probable and suspected SARS sera collected during and after the SARS outbreak. Serum samples were collected from patients admitted to the hospital during and after the SARS outbreak. Each patient was classified as either a suspect or probable case after evaluation based on Health Canada case definitions (Table 2) (http://www.hc-sc.gc.ca/pphb-dgspsp/sars-sras/sarscase def-0317_e.html). Sera were established to be positive or negative for SARS-

Table 1: Summary of patient sera

Sera	No. Patients	No. Sera	Collection date	ELISA/IFA Results (with SARS-CoV cell lysate)
Antibody Response Study				
Non-SARS	100	100	2000-2001	-
SARS Positive	132	194	2003	Positive
Paired Patient				
Acute[1]	68	84	2003	Positive
Convalescent[2]	85	110	2003	Positive
Specificity Study				
Non-SARS	190	190	2000-2001	-
SARS Negative	228	441	2003	Negative
SARS Positive	149	233	2003	Positive
Paired Patient	100	200		
Acute[1]	50	100	2003	Negative
Convalescent[2]	50	100	2003	Positive

[1] Sera collected from days 1 to 28 post onset of illness
[2] Sera collected > 28 days post onset of illness

Table 2: Severe acute respiratory syndrome (SARS) case definitions (Health Canada)

Suspect case

1) A person presenting after 1 November 2002, with:
   - Early clinical presentation of SARS, i.e.:
     - high fever (>38°C) **AND** cough or breathing difficulty

   AND
   - One or more epidemiological links to a person or place linked to SARS, during the 10 days prior to the onset of symptoms, i.e.:
     - close contact[1] with a person who is a suspect or probable case of SARS
     - history of travels to an affected area
     - residing in an affected area

2) A deceased person with:
   - unexplained acute respiratory illness resulting in death after 1 November 2002, but on whom no autopsy has been performed

   AND
   - One or more epidemiological links to a person or place linked to SARS, during the 10 days prior to the onset of symptoms, i.e.:
     - close contact[1] with a person who is a suspect or probable case of SARS
     - history of travels to an affected area
     - residing in an affected area

Probable case

A suspect case with:
   - Radiographic evidence consistent with SARS, i.e.:
     - infiltrates consistent with pneumonia or respiratory distress syndrome (RDS) on chest X-ray (CXR)

   OR
   - Laboratory evidence of SARS-CoV infection, i.e.:
     - positive results for SARS coronavirus by one or more assays (ex. PCR positive, or seroconversion, or virus isolation)

   OR
   - Autopsy findings consistent with SARS, i.e.:
     - pathology of RDS without an identifiable cause

Exclusion criteria

A case should be excluded if an alternative diagnosis can fully explain their illness

[1] Close contact means having cared for, lived with or had direct contact with respiratory secretions and/or body fluids of a person with SARS.

CoV by immunofluorescence assay and enzyme-linked immunosorbent assay (ELISA) using SARS-CoV cell lysate.

In the Antibody Response study, a paired patient serum is defined as a SARS patient with a positive acute (sera collected from days 1 to 28 post onset of illness) and positive convalescent-phase sera (sera collected >28 days post onset of illness). Alternatively, paired patient sera in the Specificity study consisted of a SARS patient with a negative acute-phase serum and a positive convalescent-phase serum.

All human sera were γ-irradiated prior to use.

2.3 Antibodies and Primers

See Table 3 for a list of primary and secondary antibodies used.

See Appendix I for a list of primers used. Primers for Tor2, OC43, and 229E CoVs are based on Genbank sequences NC_004718, NC_005147, and NC_002645, respectively.

2.4 RNA Extraction

RNA extraction was performed to generate RNA template for RT-PCR. Vero E6 cells were infected with Tor2 CoV at a M.O.I of ~1. Cells were harvested at 3 days post infection. Total RNA was extracted using the Qiagen RNeasy MINI Kit (Qiagen). Briefly, isolation of RNA was performed in the following manner: 1) lyse infected cells with guanidine isothiocyanate-containing buffer; 2) homogenize infected cells by vortexing; 3) adsorption of RNA to column with the addition of 70% ethanol; 4) RNA wash with 96% ethanol and 5) elute RNA in RNase-free water.

Table 3: Summary of primary and secondary antibodies

Antibody	Company	Source	Method	Dilution
Primary Antibodies				
Anti-his	Qiagen	Mouse	Western Blot	1:2000
			ELISA	1:100 - 1:3200
Human sera		Human	Western Blot	1:500
			ELISA	1:100 - 1:3200
Secondary Antibodies				
Anti-mouse IgG HRP	Pierce	Goat	Western Blot	1:20,000
			ELISA	1:10,000
Anti-human IgG HRP	Pierce	Goat	Western Blot	1:30,000
			ELISA	1:10,000
Anti-human IgM HRP	KPL	Goat	Western Blot	1:10,000
			ELISA	1:2500
Anti-human IgA HRP	KPL	Goat	Western Blot	1:5000
			ELISA	1:2500

2.5 Polymerase Chain Reaction (PCR)

PCR was performed on pBlueBac4.5 SARS-N, pBlueBac4.5 OC43-N, and pBlueBac4.5 229E-N clones to generate C- and N-terminal constructs. PCR was performed using Expand High Fidelity PCR System (Boehringer Mannheim) in a GeneAmp PCR System 9700 thermocycler (Applied Biosciences). This system was chosen as it is composed of a unique enzyme mix containing thermostable *Taq* DNA and *Pwo* DNA polymerases that produces PCR products with high yield, high fidelity and high specificity. Two mastermixes were set up (total volume of 25 µl each), then combined. Mastermix 1: 11 µl of sterile water, 1 µl of dNTP (10 mM each), 1.5 µl of 10 µM forward primer, 1.5 µl of 10 µM reverse primer, and 10 µl of DNA (1ng to 10 ng). Mastermix 2: 5 µl of 10x PCR buffer with 15 mM $MgCl_2$, 19 µl of sterile water, 1 µl enzyme mix. All reactions were set up on ice.

To screen for the presence of correct clones, PCR was performed using *Taq* DNA Polymerase (Sigma). A typical 50 µl reaction consisted of: 32 µl of sterile water, 5 µl of 10x PCR buffer, 1 µl of dNTP (10 mM each), 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 10 µl of DNA (1ng to 10 ng). All reactions were set up on ice.

A typical thermocycling program for PCR were as follows:

Cycles	Step	Time	Temperature
1x	Initial denaturation	2 min	94°C
35-50x	3-step cycling:		
	Denaturation	30 sec	94°C
	Annealing	30 sec	~ 5°C below T_m of primers
	Extension	1-2 min	72°C
1x	Final extension	10 min	72 °C
∞	Hold		4°C

2.5.1 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR was accomplished using Qiagen OneStep RT-PCR Kit (Qiagen) and performed in a GeneAmp PCR System 9700 thermocycler (Applied Biosciences). See Appendix I for a list of inserts that were generated by RT-PCR. A typical 50 µl reaction consisted of: x µl RNase-free water, 10 µl of 5x RT-PCR buffer, 2.0 µl dNTP (10 mM each), 3 µl of 0.6 µM forward primer, 3 µl of 0.6 µM reverse primer, 0.5 µl of RNase inhibitor, x µl DNA (~ 1 µg), and 2.0 µl of enzyme mix. All reactions were set up on ice. A typical thermocycling protocol for RT-PCR included:

Cycles	Step	Time	Temperature
1x	Reverse transcription	30 min	50°C
1x	Initial denaturation	15 min	95 °C
25-40x	3-step cycling:		
	Denaturation	30 sec	94°C
	Annealing	30 sec	~ 5°C below T_m of primers
	Extension	1-2 min	72°C
1x	Final extension	10 min	72 °C
∞	Hold		4°C

2.5.2 Product analysis

All amplicons were verified for size and quality by 1-2% agarose gel electrophoresis, run in Tris/Borate/EDTA (0.89 M Tris base, 0.89 M Boric acid, 0.02 M EDTA, pH 8.0) electrophoresis buffer. Agarose gels were stained with ethidium bromide, which was added to the melted agarose when casting the gel. The gels were run at 100 volts for 30 minutes and DNA was visualized with AlphaEase FluoroChem 5500 (Alpha Innotech).

2.6 Cloning

Using sequence specific primers (Appendix I), inserts were generated either by PCR or RT-PCR and verified by 1-2% agarose gel electrophoresis. Amplicons were PCR purified (QIAquick PCR purification kit, Qiagen) or gel extracted (QIAquick gel extraction kit, Qiagen) and cloned into pBlueBac4.5, an insect expression vector. Using TOPO TA Expression Kit (Invitrogen), a typical cloning reaction consisted of: x µl H_2O, 1 µl salt solution, 1 µl TOPO vector, x µl insert. The cloning mix was incubated for 5 min at room temperature. *E. coli* TOP 10 chemically competent cells were thawed on ice and transformed by adding 2 µl of the cloning reaction. Following incubation on ice for 30 minutes, cells were heat shocked at 42°C for 30 seconds, and then placed on ice. Room temperature SOC medium was added and the reaction was incubated at 37°C with shaking for 1 hour. The reaction was then plated on LB+Amp (100 µg/ml) plates and incubated overnight at 37°C. Colonies were screened by miniprep analysis (QIAprep Spin Miniprep Kit, Qiagen) and restriction enzyme digestion. All constructs were sequenced using the dideoxy technique based on Sanger *et al.* (1977). The amplicons were analyzed using an ABI 3100 Genetic Analyzer.

2.6.1 Baculovirus Expression System (BES)

The Baculovirus Expression System allows the protein of interest to be processed in eukaryotic cells, producing a recombinant protein that resembles its native form. The baculovirus transfer vector, pBlueBac4.5, contains an *E.coli* origin of replication, an ampicillin resistance marker, a polyhedrin promoter, a cloning region downstream from the promoter in which to insert foreign genes, a large tract of AcMNPV sequence flanking the cloning region to facilitate homologous recombination, a 6xHis tag, and a *lac*Z sequence

which allows for easy selection of blue, recombinant plaques (Figure 7). Co-transfection of the transfer vector, containing the gene of interest, with baculovirus DNA, into insect cells enables recombination between homologous sites to produce recombinant viruses (Figure 8). Recombinant proteins can be produced at levels ranging between 15 and 50% of total insect cell protein.

2.6.2 SARS, OC43, and 229E CoV full-length protein constructs

Tor2 RNA was used to amplify the SARS-N (nucleotides 28,120-29,388), M (nucleotides 26,398-27,063), and E (nucleotides 26,117-26,347) ORFs by RT-PCR using sequence specific primers (Appendix I). Inserts were cloned into the baculovirus expression vector, pBlueBac4.5, such that is was in frame with the C-terminal 6xHis tag.

SARS-CoV spike gene construct was graciously provided by Darwyn Kobasa and Tracy Taylor (Respiratory Viruses, National Microbiology Laboratory) and the SARS-N construct was kindly provided by Nathalie Bastien, Diane Ward, and Shari Rietmier (Respiratory Viruses, National Microbiology Laboratory).

2.6.3 SARS, OC43, and 229E CoV truncated N protein constructs

Fragments of the N gene were cloned in frame, downstream of the polyH promoter, into the eukaryotic expression vector pBlueBac4.5. Strategies for the construction of the N protein fragments are illustrated in Figure 9. The truncated SARS-N constructs (SARS-N-Nt and SARS-N-Ct) were amplified from the full-length nucleocapsid Tor2 CoV protein by using the forward primer and the reverse primers listed in Appendix I. Similarly, OC43-N truncated constructs (OC43-N-Nt and OC43-N-Ct) were amplified from BlueBac4.5-OC43-N, while truncated 229E-N constructs (229E-N-Nt and 229E-N-Ct) were produced from pBlueBac4.5-229E-N.

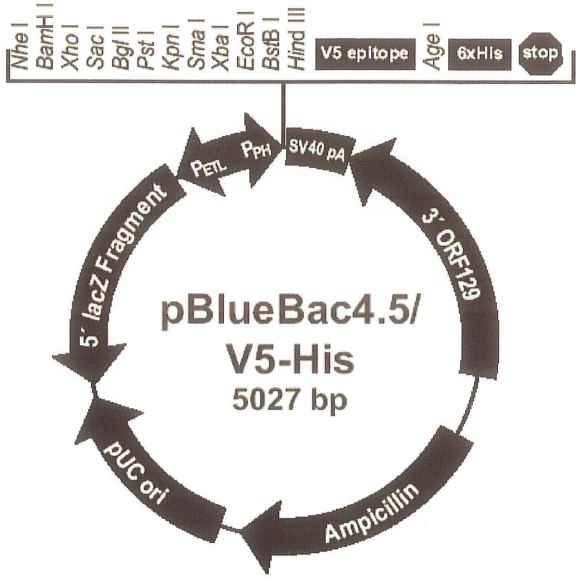

Figure 7. Vector map of pBlueBac4.5 (Invitrogen)

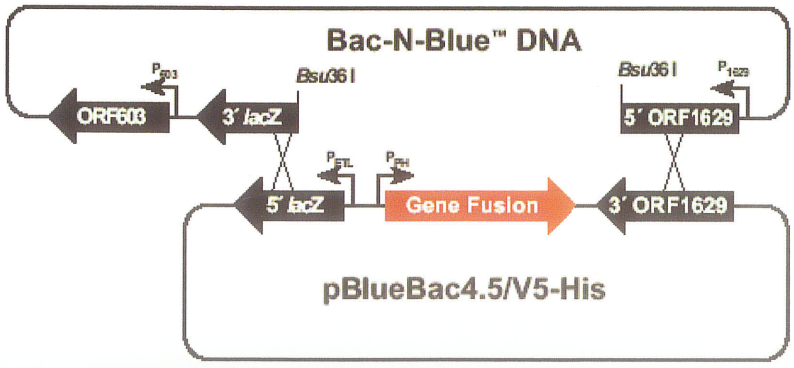

Figure 8. Recombination of transfer vector (pBlueBac4.5/V5-His) with Bac-N-Blue baculovirus DNA (Invitrogen)

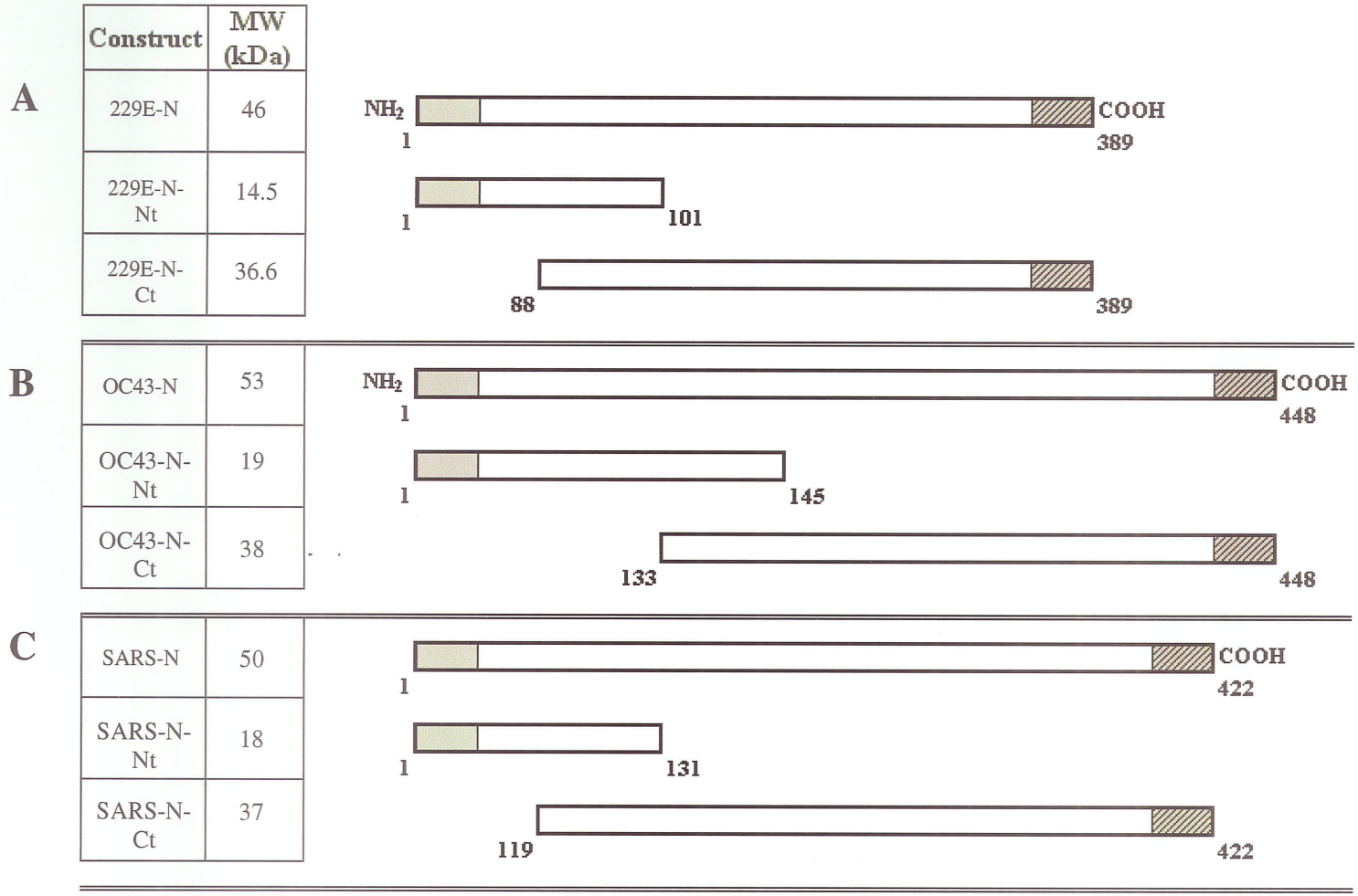

Figure 9. Schematic representation of truncated HCoV N protein constructs. The 229E (A), OC43 (B), and SARS (C) CoV constructs are listed on the left with their corresponding predicted molecular weights. The amino acids are listed below the constructs. The stop codon was removed from all constructs and a start codon was added to all C-terminal fragments.

2.7 Transfection of pBlueBac4.5 constructs into Sf21 cells

Spodoptera frugiperda 21 (Sf21) cells were seeded at ~60% confluency in 6-well plates using Serum-free medium (SF-900 II, Gibco). A recombinant transfer vector and viral DNA were co-transfected into insect cells using Cellfectin (Invitrogen). The transfection mixture was prepared by adding 1 µg of recombinant transfer plasmid, x µl SFM and 10 µl of Cellfectin to 0.5 µg of Bac-N-Blue baculovirus DNA (Invitrogen). Following a 15 minute incubation at room temperature, 800 µl of SFM was added to the mixture. Medium was then removed from Sf21 cells and the transfection mix was added. Cells were incubated for 4 hours at 27°C followed by the removal of the transfection mix and addition of 2 ml Grace's insect cell medium containing 10% FBS and 1% penicillin/streptomycin/kanamycin. Cells were incubated at 27°C for 4-6 days.

2.8 Plaque Assay

Plaque assay was used to plaque purify recombinant virus, or to determine viral titer in pfu/ml. Sf21 cells were seeded in 6-well plates at ~50% confluency. Stock recombinant virus was serially diluted 10-fold. Medium was removed from the cells and 500 µl of the 10^{-2}, 10^{-3}, and 10^{-4} dilutions were added to individual wells in duplicate. Cells were incubated for 1 hour at room temperature, during which time the plates were rocked every 15 minutes to prevent the cells from drying out. Following the incubation, the inoculum was removed and overlayed with 2 ml of warmed medium/sea plaque agarose (3%) mix to "pin down" the infected monolayers. X-gal was added to the medium to a final concentration of 150µg/ml prior to adding the agarose in order to detect blue, recombinant plaques. After cooling, 3 ml of Grace's insect cell media, containing

10% FBS and 1% penicillin/streptomycin/kanamycin, was added. Cells were incubated for 5-6 days at 27°C.

2.9 Infection of Sf21 cells

2.9.1 Stock infection of Sf21 cells with recombinant baculovirus

Sf21 cells were infected at 60-70% confluency in a T75 flask. Subsequent to removal of the media from the cells, 500 µl (M.O.I. < 1 pfu) of recombinant baculovirus or wild-type baculovirus (Wt, AcMNPV) supernatant was added. Recombinant virus was allowed to adsorb for 1 hour at room temperature. The flasks were rocked every 15 minutes to prevent cells from drying out. Following incubation, the inoculum was removed and 20 ml of Grace's insect cell media supplemented with 10% FBS and 1% penicillin/streptomycin/kanamycin was added. Cells were incubated at 27°C for 72-96 hours. Viral titer was determined using plaque assay procedure described previously. Generally, a titer of 2×10^7 pfu/ml was attained.

2.9.2 Infection for the expression of recombinant proteins

Sf21 cells were seeded in a 6-well plate or T75 flask at a confluency of 70-80%. Cells were infected at an M.O.I of ~5 pfu/cell and virus was allowed to adsorb for one hour at room temperature. After incubation, the inoculum was removed and 2ml or 20ml of media were added to a 6-well plate, or T75 flask, respectively. Cells were incubated at 27°C for 48-72 hours. The presence of the full-length copy of the SARS-N gene in the recombinant viral genome was confirmed by PCR and sequence analysis of recovered virus. Following plaque purification the recombinant viruses were amplified to obtain high titer.

2.10 Light Microscopy

Transfected or infected cells were viewed in the tissue culture dish using an Axiovert25light microscope (Zeiss).

2.11 Harvesting of Virus and Cells

2.11.1 Harvest plaque purified virus

To harvest plaque purified virus the media overlay was removed from the agarose. A single plaque was picked using a 200 µl pipette (Gilson). The resulting virus plug was added to 3 ml of Grace's insect cell media and stored at 4 °C overnight to allow the virus to diffuse from the agarose.

2.11.2 Harvest transfected or infected Sf21 cells

Transfected or infected cells and supernatants were collected and separated by centrifugation for 5 minutes at 3000 rpm in an Allegra 21R Centrifuge (Beckman Coulter). If supernatant was desired it was stored at 4°C, if not, it was discarded. The cells were then washed once with cold phosphate-buffered saline and cells were pelleted by low-speed centrifugation (3000 rpm, 5 min). Subsequent treatment was dependent upon method of analysis. For Western Blot analysis, the cell pellet was resuspended in distilled water, and an equal volume of 2X sample buffer. The cell lysates were heated for 5 min at 95°C and then stored at -20°C. For ELISA analysis, antigens were produced as described below.

2.12 Cell Lysis

To prepare recombinant proteins for use as antigen in ELISA analysis, harvested cells were resusupended in 1.0 ml of ice-cold Insect cell lysis buffer (BD Biosciences) containing protease inhibitor cocktail (BD Biosciences). Using a 23-gauge needle and a 1 ml syringe, the cells were passed through the syringe 10 times to shear the DNA. The cells were briefly vortexed and incubated on ice for 45 minutes. The lysate was cleared from cellular debris by centrifugation at 14,000 x g (Eppendorf Micro Centrifuge 5415 D, VWR) for 15 minutes at 4°C. The cleared supernatant was then harvested.

2.13 Protein Purification

Purification of the His-tagged recombinant proteins was based on the high affinity of the 6xHis tag for Ni-NTA Agarose. The 6xHis tag is a valuable tool as it is very small in size and uncharged under physiological pH conditions. In addition, it is not immunogenic and does not alter the folding, compartmentalization or biochemical properties of the recombinant protein. Furthermore, it allows for easy identification of the recombinant protein. For purification, ten volumes of insect cell lysate, containing the recombinant 6xHis fusion protein, was mixed with 1 volume of Ni-NTA Agarose (BD Biosciences). The slurry was incubated for 1 h at 4°C, on a rotating platform. Following this period of time, the slurry was microcentrifuged at 500 x g for 5 minutes to sediment the matrix. The supernatant was drawn off and the beads were washed 4 times with 6xHis Wash Buffer (BD Biosciences). To elute the recombinant proteins, the beads were incubated with varying amounts of imidazole (0.1M, 0.3M and 0.5M) in 6xHis elution (BD Biosciences) buffer for 2 minutes at room temperature on a rotating platform. The

slurry was then centrifuged at 500 x g for 5 minutes and the eluted fractions were collected. The elution process was repeated two more times at each imidazole concentration and all eluted fractions were pooled.

2.14 Protein Quantification

2.14.1 Protein quantification of infected cell lysate

Protein quantification was performed using the Micro BCA Protein Assay Kit (Pierce). The test is based on bicinchoninic acid for the colorimetric detection and quantification of total protein. Briefly, a set of diluted protein standards were prepared and assayed alongside the unknowns in order to determine the concentration of the unknown proteins from the standard curve. Initially, working reagent was prepared by mixing 25 parts of BCA Reagent A (sodium carbonate, sodium bicarbonate, bicarbonate and sodium tartrate in 0.2 M NaOH) and 24 parts of BCA Reagent B (4% bicinchoninic) with 1 part of Reagent C (4% cupric sulfate). BSA protein standards were prepared at an initial concentration of 2000 µg/ml and were serially diluted (1:2) with PBS to a final concentration of 31.3 µg/ml. To determine protein concentration, 125 µg of antigen was added to 875 µl of 1X PBS in a 2ml deep-well plate. The antigen was serially diluted (1:2) using 1X PBS. Then 100 µl of each sample and the standard were aliquoted in triplicate into a 96-well microplate (Nunc Maxisorp 96-well plate, VWR). The working reagent was added, 100 µl to each well, and incubated for 1.5 hrs at 37°C. The absorbance was measured at 562 nm on a Microplate Reader 550 (Biorad). A standard curve was prepared by plotting the average blank 562 nm reading for each BSA standard

versus its concentration in µg/ml. From the standard curve the protein concentration of each unknown sample was determined.

2.14.2 Protein quantification of immunoblot protein bands

The band intensites of expressed proteins was measured on a Typhoon 9410 (Molecular Dynamics) using ImageQuant software. Briefly, protein expression levels were determined by volume quantification. In this method, the volume of each band was obtained by subtracting a set background value from the measured intensity of each protein band. The volume was then used to compare band intensities by taking the volume of a single band as a percentage of the total volume of all protein bands.

2.15 SDS-PAGE and Wet Transfer

Proteins were separated on 10 cm (l) x 7.5 cm (h) (thickness 1.0 mm) 12% SDS mini gels by electrophoresis (150 volts for 1.5 hours) in a Mini Protean 3 Electrophoresis Cell (Biorad). A discontinuous buffer system that incorporates SDS in the buffer was used to ensure high resolution of the proteins (Laemmli, 1970). In this system, proteins are first denatured by heating them in a buffer that contains sodium dodecyl sulphate (SDS) and a thiol reducing agent, such as 2-mercaptoethanol, before being loaded on a gel. The denatured proteins will then migrate through the gel according to their molecular weight. Following separation, proteins were transferred from the SDS-polacrylamide gel to a nitrocellulose transfer membrane (Biorad) using a Trans-blot electrophoretic wet transfer cell (Biorad).

Wet transfer for a mini-gel was performed by placing one side of the gel in contact with a piece of nitrocellulose membrane. The gel and the attached membrane

were then sandwiched between filter paper, two porous pads, and closed within a plastic cassette. As each layer was added, a pipette was rolled over the surface to remove any air bubbles. The entire construct was immersed in an electrophoresis tank, containing tris-glycine electrophoresis buffer (250 mM-Tris, 1.92 M-glycine, 0.2% methanol). Transfer of a mini-gel was run at 100 V, for 60 minutes.

2.16 Immunoblot

Following transfer, the membrane was blocked overnight in 5% skim milk powder in TBS buffer (150 mM NaCL, 10 mM Tris-HCL, pH 7.5) to reduce non-specific binding to the membrane. The following morning, the blot was incubated with the primary antibody for 1 hour at room temperature on a rotating platform. The blot was then washed three times with TT-TBS (0.05% Tween20, 0.2% Triton-X, 150mM NaCL, 10mM Tris-HCL, pH7.5) and then incubated with the secondary antibody for 1 hour at room temperature on a rotating platform. The blot was again washed with TT-TBS four times, and proteins were visualized using the Supersignal Dura West detection system (Pierce). This sensitive chemiluminescent method is based on an antibody system coupled to horseradish peroxidase, which converts an ECL substrate into a light signal. Briefly, reagent A and B were mixed in equal volumes and 3 ml were added to each membrane and incubated at room temperature for 2 minutes. The membranes were exposed to Fugi Rx-Film (Fisher Scientific) in the dark and developed on Feline 4 X-ray film processor (Fischer).

2.16.1 Competition immunoblot assay

The competition assay was used to investigate the potential cross-reaction between human coronaviruses and was performed similar to the procedure described above. Prior to immunoblotting, human sera was diluted in TBS (1:500) and incubated with 120 µg of SARS-N, OC43-N, or 229E-N antigen for 1 hour on a rotating platform. The antibody/antigen mixture was then incubated with the membrane for 1 hour at room temperature. Subsequent steps were followed as described above.

2.17 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was performed to quantify the amount of antibodies produced in response to each recombinant CoV protein. The assay was based on the amount of colour development, which is proportional to the amount of antibody bound. Antigens were prepared by infecting monolayers of Sf21 cells with the appropriate recombinant baculovirus as described above. After 72 hours the cells were harvested and lysed. Proteins in the cell lysate were quantified and diluted to 3 mg/ml with PBS. Wild-type baculovirus (AcMNPV) infected cells were used as a background control for each sera. Subsequently, each well of an immunoplate (Nunc Maxisorp 96-well plate, VWR) was coated with His-tagged recombinant protein (3 µg/ml per well) at 4°C overnight and then blocked in phosphate buffered saline with 10% bovine serum albumin. The plates were then washed on an automated plate washer (Skan Washer 300, Molecular Devices) with wash buffer (150 mM NaCl, 0.05% Tween20, 0.01% Thimersol, PBS, pH 7.5) for 3 cycles and blotted on paper towels. Human sera, diluted in serum diluent (0.2% Tween20, 10% BSA, PBS, pH 7.5) were added to the wells of the recombinant protein-

coated plates in a total volume of 100 µl, and the plates were incubated at room temperature for 2 h. After the plates were washed 5 times with wash buffer, 100 µl of the secondary antibody was diluted in PBS-Tween and added to each well. Following a 1.5 hour incubation at room temperature, the plates were washed five times with PBS-Tween, and colour development was carried out by the addition of 100 µl of the enzyme substrate teteramethylbenzidine (TMB, Pierce) per well. After a 1 hour incubation at room temperature in the dark, the reaction was stopped by adding 100 µl of 0.6 M H_2SO_4 per well. Optical densities (OD) were measured at 450 nm with an automated ELISA reader (Microplate Reader Model 550, Biorad). The corrected OD value for each protein was obtained by subtracting the control (AcMNPV infected) lysates from all values.

2.17.1 ELISA for detection of antibodies against CoV proteins

Microtiter plates coated with recombinant OC43-N, 229E-N, or SARS-CoV nucleocapsid, spike, matrix, or envelope protein (3 µg/ml per well) were blocked for 1 hour and then washed 3 times. Diluted human sera (1:100) were then added and incubated for 2 hours at room temperature. Following 5 plate washes, 100 µl of diluted horseradish peroxidase-conjugated goat anti-human IgG (1:30,000, Pierce) was added to the wells. The plates were then incubated for a further 1.5 hours at room temperature and washed 5 times. Then 100 µl of TMB was added per well and incubated for 1 hour in the dark, at room temperature. The reaction was stopped by adding 0.6 M H_2SO_4 per well and the absorbances were read at 450 nm. The OD value obtained for control (AcMNPV infected) lysates was subtracted from all values.

2.17.2 Longitudinal profile of IgG, IgM, and IgA antibodies against SARS-CoV nucleocapsid protein

SARS-CoV N coated plates were blocked for 1 hour and then washed 3 times. Human sera were serially diluted 2-fold in serum diluent, starting from a dilution of 1:100 to 1:3200, and then added to the plates. After a 2 hour incubation at room temperature, the plates were washed 5 times and 100 µl of diluted horseradish peroxidase-conjugated goat anti-human IgG (1:30,000, Pierce), goat anti-human IgA (1:5,000, KPL), or goat anti-human IgM (1:10,000, KPL) antibodies were added to the wells. Following a 1.5 hour incubation at room temperature, the plates were washed 5 times and then 100 µl of TMB was added to each well. The plates were incubated at room temperature for 1 h and the reaction was stopped using 0.6 M H_2SO_4. The absorbance at 450 nm of each well was measured. To obtain corrected values, the control OD values were subtracted from tested values. The antibody titer was defined as the highest dilution of the serum, which had an absorbance at 450 nm wavelength greater than the cutoff value.

2.18 Serum Neutralization Assay

Serum specimens were tested for neutralizing activity as follows. The serum samples were heated for 30 minutes at 56°C and serially diluted from 1:8 to 1:16384 with MEM prior to mixing with 100 $TCID_{50}$ of SARS-CoV (Tor2). After incubation overnight at 4°C, the mixture was inoculated, in duplicate, into 96-well plates of Vero E6 cells for 1 hour at room temperature. Finally, 100 µl of virus growth media (MEM containing 2% FBS, 25 mM HEPES buffer, 0.1 mM MEM Non-Essential Amino Acid solution, 1.0 mM MEM Sodium Pyruvate solution and 1% penicillin/streptomycin/ glutamine) was added

to each well and incubated for 5 days at 37°C and 5% CO_2. The titers were expressed as the reciprocal of the highest dilution at which the CPE was completely inhibited. Serum from a non-SARS patient was used as a negative control, while serum from a confirmed SARS patient was used as a positive control. Neutralizing assay was performed in duplicate.

3 RESULTS

3.1 Immune Response Elicited to SARS-CoV Infection

To study the humoral response during SARS-CoV infection, the antibodies produced specifically to the structural proteins of SARS-CoV were evaluated by ELISA and Western blotting.

3.1.1 Expression of pBlueBac4.5 SARS-CoV constructs

To gain insight into the primary targets of the immune response after SARS-CoV infection, the main structural proteins of SARS-CoV were expressed in insect cells using a baculovirus expression system. At 72 hours post infection, the cells were harvested, lysed, and protein was quantified. Although SARS-CoV N was highly expressed, SARS-CoV M and E were subject to purification, based on the presence of the His tag, in order to increase protein concentration. The SARS-CoV N and S cell lysate and purified M and E proteins were then analyzed for the expression of SARS-CoV proteins by immunoblot using mouse antibodies against the His-tagged proteins (1:2000) followed by a goat anti-mouse HRP-conjugated antibody (1:20,000). Immunoblot analysis revealed bands at 180 kDa, 55 kDa, 28 kDa, and 9 kDa indicative of recombinant SARS-CoV S, N, M, and E proteins, respectively (Figure 10). The negative controls included uninfected cells, and wild-type baculovirus (Wt, AcMNPV), which had the same treatment as the other samples (Figure 10, lane Wt and C, respectively).

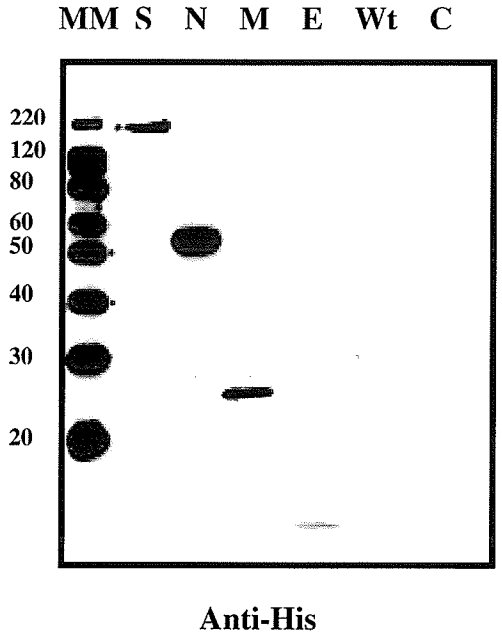

Figure 10. Western blot analysis of recombinant SARS-CoV proteins. Sf21 insect cells were infected with recombinant baculovirus expressing either the SARS-CoV S (lane S, 180 kDa), N (lane N, 55 kDa), M (lane M, 28 kDa), or E (lane E, 12 kDa) proteins. Proteins were detected with anti-His (1:2000) followed by a HRP-conjugated goat anti-mouse antibody (1:20,000). Lane MM, Magic Marker; Lane Wt, Wt (AcMNPV) infected cell control; Lane C, Sf21 cell control.

3.1.2 Detection of IgG antibodies against viral proteins encoded by the SARS-CoV genome

To investigate the antibodies produced against the main structural proteins of SARS-CoV, recombinant protein-based ELISA was employed. In order to accurately characterize the antibody response to SARS-CoV proteins by ELISA analysis, the background resulting from the non-specific binding of normal human sera to viral lysate was calibrated for each viral protein. This was accomplished by screening 100 non-SARS sera, collected prior to the SARS outbreak, to obtain the adjusted optical density (OD) values (OD of control – OD of wild-type infected cells). The cutoff values were then defined as the sum of the mean adjusted OD values from the control and three times the standard deviation to obtain the following readings for SARS-N, S, M, and E: 0.087, 0.043, 0.043, and 0.029, respectively.

Using the established cutoff values, the antibody responses to SARS-CoV proteins were evaluated from a total of 194 serum samples, obtained from 132 patients that were confirmed to be positive for SARS-CoV by IFA and ELISA using SARS-CoV infected whole cell lysate. The samples were collected from days 2 to 256 after the onset of symptoms, and tested by recombinant SARS-CoV protein specific ELISAs. Resulting profiles are shown in Figure 11 and further detailed in Table 4. The recombinant protein-based ELISA showed the greatest reactivity to the SARS-N protein (98.5%), making the detection rate of the recombinant protein-based ELISA similar to that of SARS-CoV infected cell-based ELISA. For the remaining proteins, SARS-S had the highest detection rate (73.7%), followed by SARS-E (30.9%), and SARS-M (28.3%) protein. Antibodies against the nucleocapsid protein could be detected in SARS serum as early as

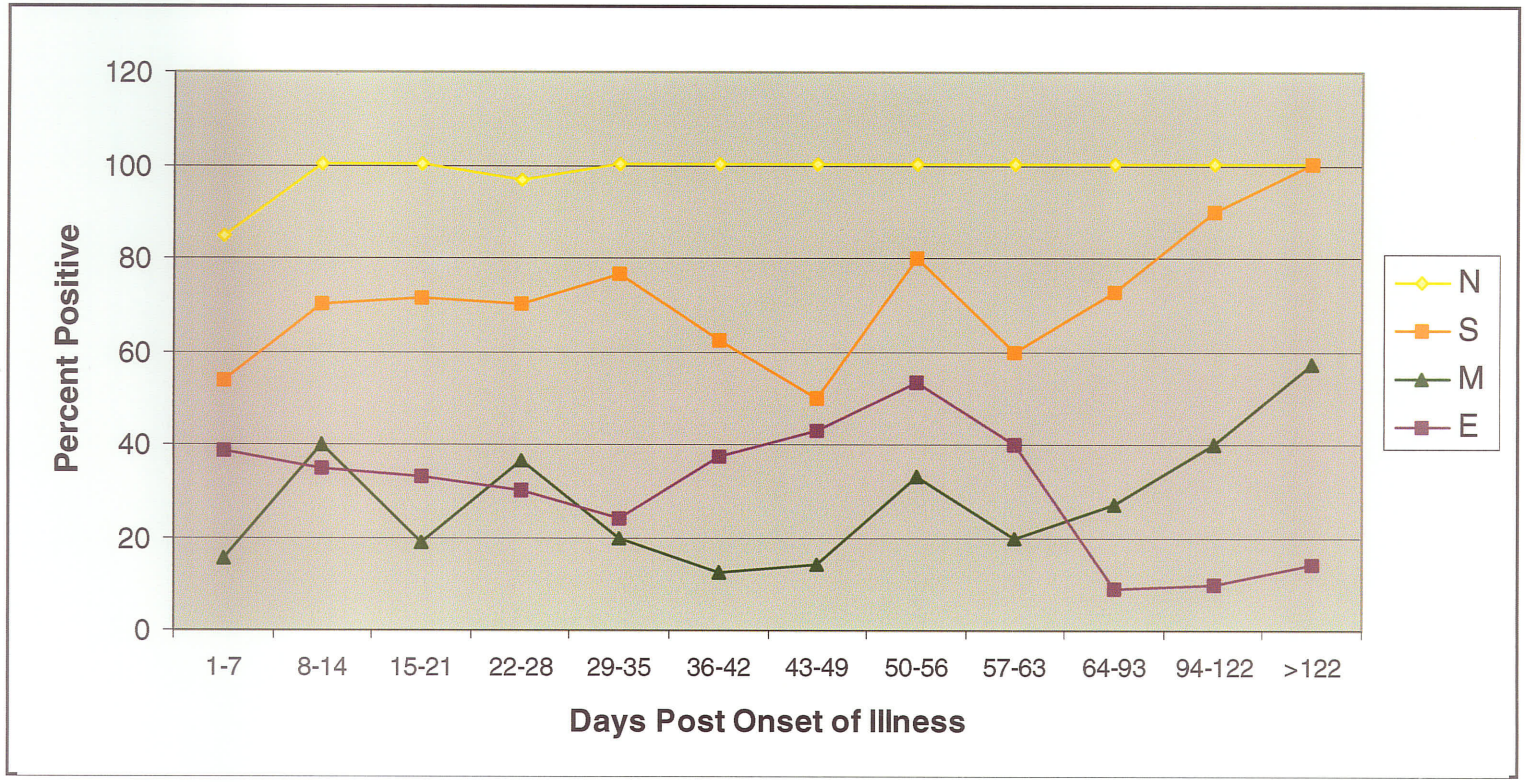

Figure 11. Antibody response to SARS-CoV structural proteins throughout the course of illness. The presence of SARS-CoV N, S, M, and E proteins were detected by recombinant protein-based ELISA.

Table 4. Detection of IgG antibodies to SARS-CoV structural proteins at various times points by recombinant protein-based ELISA.

Days post onset of illness	Samples (n)	Positives [n (%)]							
		N		**S**		**M**		**E**	
1-7	13	11	(84.6)	7	(53.8)	2	(15.4)	5	(38.5)
8-14	20	20	(100.0)	14	(70.0)	8	(40.0)	7	(35.0)
15-21	21	21	(100.0)	15	(71.4)	4	(19.0)	7	(33.3)
22-28	30	29	(96.7)	21	(70.0)	11	(36.7)	9	(30.0)
29-35	25	24	(100.0)	23	(76.7)	5	(20.0)	6	(24.0)
36-42	16	16	(100.0)	10	(62.5)	2	(12.5)	6	(37.5)
43-49	14	14	(100.0)	7	(50.0)	2	(14.3)	6	(42.9)
50-56	15	15	(100.0)	12	(80.0)	5	(33.3)	8	(53.3)
57-63	5	5	(100.0)	3	(60.0)	1	(20.0)	2	(40.0)
64-93	11	11	(100.0)	8	(72.7)	3	(27.3)	1	(9.1)
94-122	10	10	(100.0)	9	(90.0)	4	(40.0)	1	(10.0)
>122	14	14	(100.0)	14	(100.0)	8	(57.1)	2	(14.3)
Total	194	191	(98.5)	143	(73.7)	55	(28.3)	60	(30.9)

day 2 and until day 256 post onset of illness, and reached 100% detection rate by day 8. For spike protein, positive reactions were observed from days 3 to 256 after the onset of illness and the greatest response (100%) was achieved at >122 days post onset of disease. Antibodies to SARS-CoV M and E proteins were first evident at days 5 and 3, respectively, and both remained detectable until day 252 post onset of illness. SARS-CoV M attained a maximum response of 57% at >122 days post symptom onset, while SARS-CoV E response peaked at 53% at days 50 to 56 post onset of illness. Given that data on the day of collection post onset of illness was available for all of the sera tested, the average day of seroconversion for each protein was determined to be 47.8, 43.4, 55.3, and 35.8 for SARS-CoV S, N, M, and E, respectively. As the detection of antibodies against SARS-CoV N was the earliest and greatest of all SARS-CoV structural proteins the recombinant SARS-CoV N protein-based ELISA would provide the best diagnostic tool.

To further assess the reactivity of serum samples against the viral proteins encoded by the SARS-CoV genome, Western blot analysis was performed on selected SARS positive sera. The results of the immunoblots revealed the presence of antibodies against the recombinant N protein in all 44 SARS sera tested (Figure 12B, lane N), whereas, far fewer patients (37.9%) reacted to the S protein of SARS-CoV, the majority of which had sera collected late in the convalescent phase (Figure 12B, lane S). There was an even lower detection of antibodies against the M protein in SARS patient sera (5%), while the E protein showed no immunoreactivity to any of the SARS samples tested (Figure 12B, lane M and E, respectively). None of the patient sera reacted with the

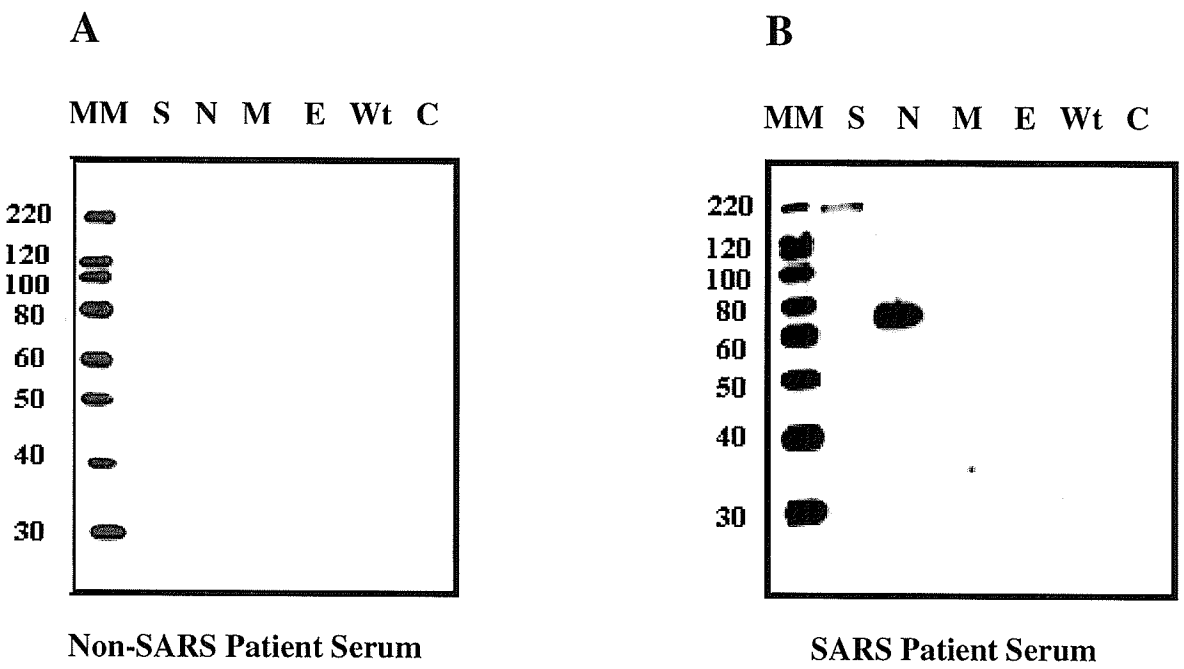

Figure 12. Detection of antibodies against SARS-CoV structural proteins in human sera. Recombinant SARS-CoV proteins were expressed in Sf21 insect cells and probed with non-SARS (control) serum (A) and SARS patient serum (B) (1:500) followed by anti-human IgG antibody (1:30,000). Lane MM, Magic Marker; Lane Wt, Wt (AcMNPV) infected cell control; Lane C, Sf21 cell control

control, AcMNPV infected insect cells (Figure 12, lane Wt). Likewise, non-SARS patient sera did not react with any of the recombinant proteins (Figure 12A).

3.1.3 Profile of IgM, IgG, and IgA antibodies against SARS-CoV N protein

Based on the results of recombinant SARS-CoV protein-based ELISA and Western blot analysis, the nucleocapsid protein is the most immunogenic antigen. Therefore, to further characterize the humoral immune response to SARS-CoV nucleocapsid protein, the IgM, IgG, and IgA antibodies were profiled in positive SARS serum specimens by ELISA. Initially, a cutoff value was established for the assay by serially diluting serum samples (1:2) from 100 non-SARS patients, which were then screened for SARS-CoV N specific antibodies by ELISA. The cutoff values were defined as the sum of the mean adjusted values from the control serum (OD of control – OD of wild-type baculovirus infected cells) and three times the standard deviation. The following ranges corresponding to the reciprocal dilution from 100 to 3200 were obtained for IgG, IgA, and IgM: 0.081 to 0.012, 0.087 to 0.03, and 0.13 to 0.038, respectively.

Next, the appearance and persistence of IgG, IgM, and IgA antibodies to SARS-CoV was investigated in 194 serum samples ranging in collection days from 2 to 256 after the onset of illness. The resulting profiles of serially diluted serum samples tested by N protein-based ELISA are shown in Figure 13. Overall, the majority of SARS positive sera produced an anti-nucleocapsid IgG response (97.9%), followed by that of IgA (74.2%), and lastly, IgM (46.9%). In contrast to the IgG response to the N protein, which ranged in detection from day 3 to 256 days post onset of illness, the production of N specific IgM, and IgA antibodies was detected as early as day 1 from symptom onset

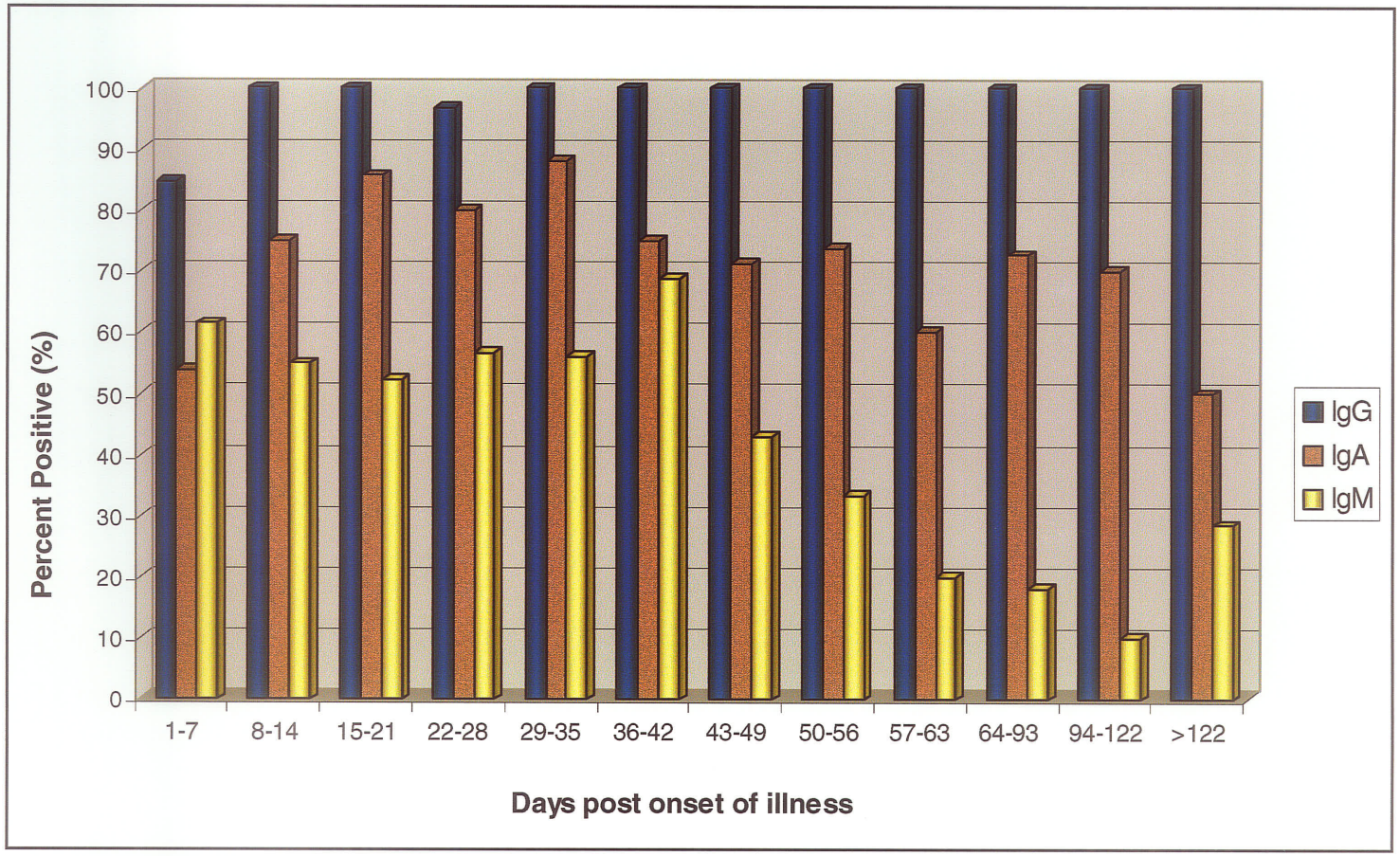

Figure 13. Response profile of various immunoglobulins to recombinant SARS-CoV N protein in human sera. SARS positive sera were serially diluted and tested for IgG, IgA, and IgM antibodies throughout the course of the disease by recombinant protein-based ELISA.

and was still detected in patients at day 198 and 256 post onset of illness, respectively. As evaluated previously, the earliest maximum IgG antibody response, which reached 100%, was attained at days 8 to 21 and remained consistently high throughout the course of illness. For IgA there was a mean peak positive response of 88% at days 29 to 35. The IgM antibodies detected against the N protein reached a maximum response of 69% at days 36 to 42, and showed at steady decline between days 42 to 122 post onset of illness. By day, 122, 100%, 50%, and 29% of the patients were still positive for IgG, IgA, and IgM specific anti-nucleocapsid protein, respectively.

Of the 194 SARS sera tested, 84 were collected from patients in the acute-phase (days 1 to 28 post onset of illness), while the remaining 110 were obtained from patients in the convalescent-phase (>28 days post onset of illness). The anti-nucleocapsid IgG antibodies were detected in 96% of acute-phase SARS sera, which increased to 100%, in convalescent-phase SARS sera. As expected, IgM had a higher detection rate in acute SARS sera (56%) than the detection rate in convalescent SARS sera (40%). The detection rate of IgA remained fairly constant at about 74% in acute and convalescent-paired patient sera. The mean time to seroconversion for IgG, IgA, and IgM, detected by ELISA, were days 44, 38, and 32 after disease onset, respectively.

By serial dilution, we were able to determine the anti-viral IgG, IgM, and IgA antibody titers of patients' sera throughout the course of disease (Figure 14). Results indicate that the IgG antibody titer exceeds that of IgA, or IgM. Anti-viral IgG attained the highest mean titer of 1:3200 late after disease onset at days 94 to 122. Lower titers of anti-viral IgA and IgM antibodies were present after the onset of illness, with IgA

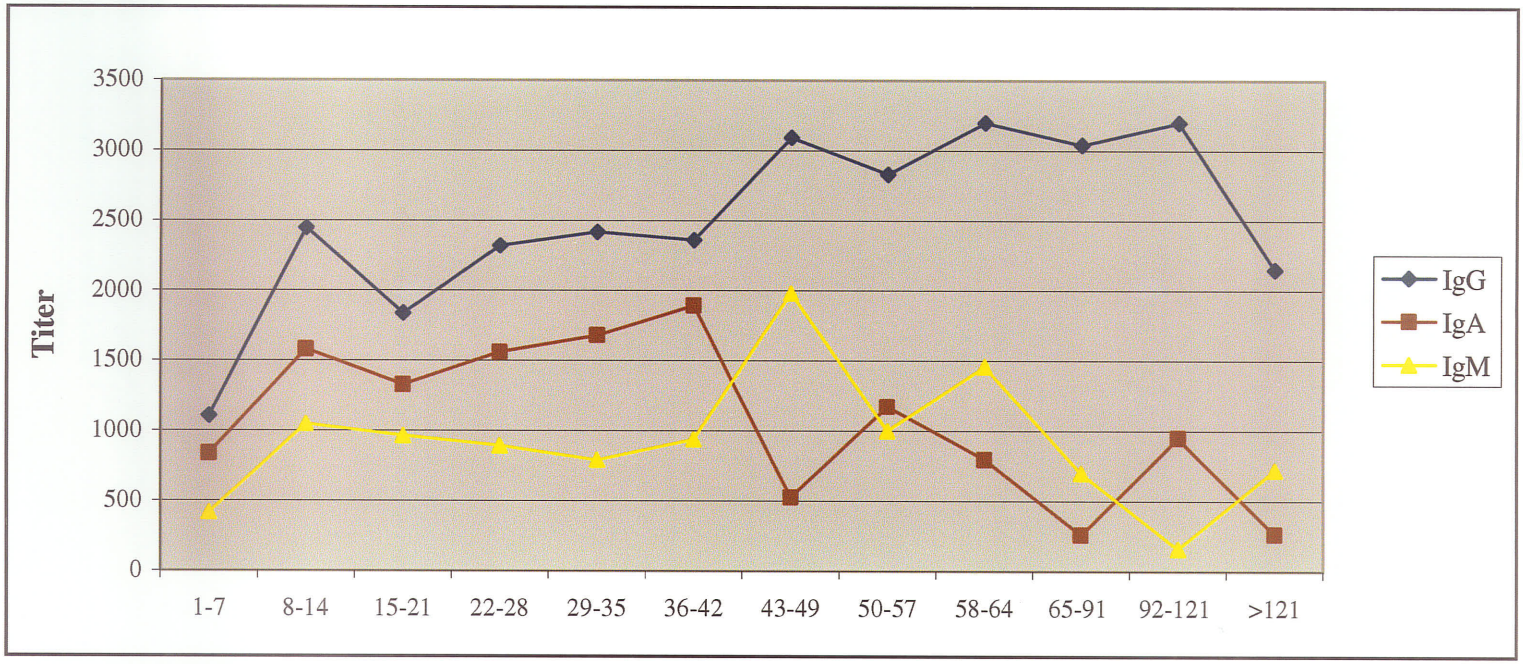

Figure 14. Changing IgG, IgA, and IgM antibody titers to the SARS-CoV nucleocapsid protein. SARS positive sera were subject to recombinant protein-based ELISA to determine the titers from the onset of illness through the convalescent phase.

reaching its peak mean titer of 1:1893 at days 36 to 42 post onset of illness, followed by IgM of 1:1980 at days 43 to 49 post symptom onset.

To detect variation in antibody production against the SARS-CoV nucleocapsid protein throughout the course of the disease, Western blot analysis was performed using SARS serum collected at different time points post illness onset. Figure 15 illustrates a typical response profile of IgG, IgA, and IgM to recombinant SARS-CoV N protein. As detected by ELISA, the SARS positive serum produced the greatest anti-nucleocapsid IgG response throughout the course of the disease, followed by IgA and IgM. The IgG, and IgM responses were initiated as early as day 3 after illness onset and progressively increased from days 18 to 35. The IgA response was detected at day 18 and remained at a high level when detected at day 35-post symptom onset. In support of the ELISA results, there was an observable rise in the IgG, IgA, and IgM specific antibodies with increasing collection day. Tor2 infected cell lysate was run as a positive control and generated multiple bands that fell below those of SARS-CoV N as a result of the additional SARS-CoV N His tag. Wild-type baculovirus infected cells and uninfected Sf21 cells were used as negative controls (Figure 15, lanes T, Wt, C, respectively).

3.2 Specificity of Serological Response to Recombinant SARS-CoV N Protein

Although the SARS outbreak has officially been declared over, the possibility for it to re-emerge exists. In the absence of effective drugs or vaccines, the first line of defence against SARS is to identify and isolate infected patients in the early stage of illness. Therefore, it is critical to develop accurate and sensitive diagnostic tools for

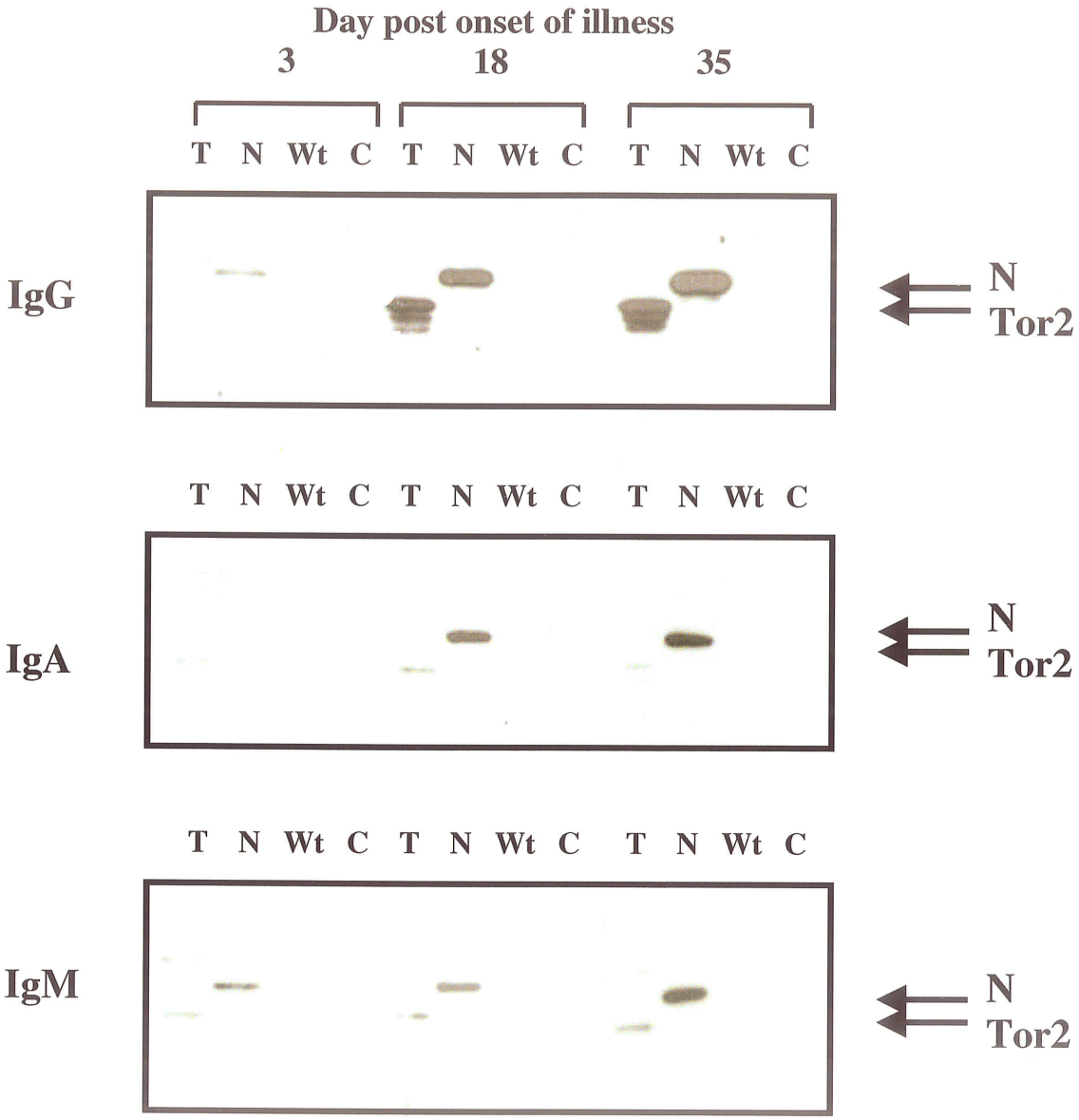

Figure 15. Detection of specific SARS-CoV nucleocapsid antibodies at 3 different time points post symptom onset. Immunoblot was incubated with patient sera (1:500) and probed with anti-human IgG (1:30,000), anti-human IgA (1:5,000), or anti-human IgM (1:10,000). Lane T, Tor2 whole cell lysate control; Lane N, SARS-CoV N protein; Lane Wt, Wt (AcMNPV)infected cell control; Lane C, Sf21 cell control.

identifying SARS. Based on the above findings, SARS-CoV N protein appears to hold great potential as a powerful and sensitive diagnostic reagent for the detection of SARS-CoV infection. However, a good diagnostic tool is not only sensitive but specific as well. With this in mind, non-SARS sera, collected at least one year prior to the SARS outbreak, and negative SARS sera were investigated for non-specific binding of antibodies to SARS-CoV N protein by recombinant protein-based ELISA.

Initially, the baseline for the test was established by analyzing, 190 non-SARS patient sera by recombinant SARS-CoV N protein-based ELISA. To control for non-specific binding to baculovirus antigens, each serum tested was assayed for binding to both wild-type baculovirus and recombinant baculovirus expressing CoV N lysate. The cutoff OD_{450} value for a positive diagnosis was designated as 0.05 above 1.5 times the background (Wt baculovirus infected cells), individually calculated for each sample.

Next, the antibody response to SARS-CoV N protein was investigated in sera collected during the SARS outbreak but deemed negative by SARS-CoV infected whole cell lysate-based ELISA and IFA, as well as in non-SARS serum samples collected at least one year prior to the 2003 SARS outbreak. By recombinant SARS-CoV N protein-based ELISA, the detection rate was 6.6% for SARS negative sera, and a surprising 2.1% for non-SARS sera (Table 5). Further analysis by serum neutralization and Western blot, verified the occurrence of false-positive ELISA results in non-SARS sera (Figure 16A, patient 1) negative SARS sera (Figure 16B, patient 2).

Table 5. Summary of ELISA results investigating the response to full-length HCoV nucleocapsid proteins in SARS positive, negative, and non-SARS sera.

Sera	Full-length Constructs	No. Positives	Total No.	% Positive
SARS POSITIVE SERA	SARS-N	233	233	100
	OC43-N	91		39.1
	229E-N	194		83.3
SARS NEGATIVE SERA	SARS-N	29	441	6.6
	OC43-N	192		43.6
	229E-N	301		68.3
NON-SARS SERA	SARS-N	4	190	2.1
	OC43-N	64		33.7
	229E-N	124		65.3

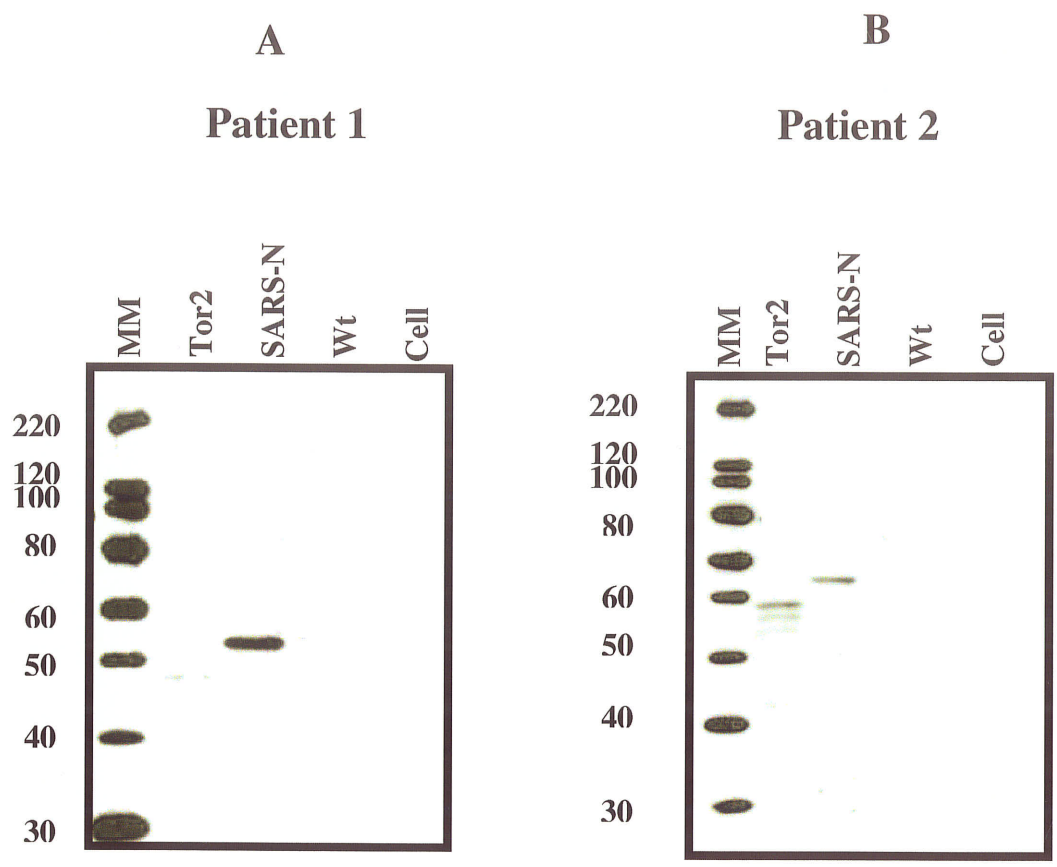

Figure 16. Immunoblot detecting false-positive samples from non-SARS serum (A, patient 1) and negative SARS serum (B, patient 2). The recombinant CoV nucleocapsid proteins were incubated with human serum (1:500) and the response was detected by goat anti-human HRP antibody (1:30,000). Lane Tor2, Tor2 whole cell lysate control; Lane MM, Magic Marker; Lane Wt, Wt (AcMNPV) infected cell control; Lane Cell, uninfected Sf21 cell control.

3.3 Cross-reactivity of anti-HCoV N antibodies

Although SARS-CoV forms a distinct group within the genus *Coronavirus*, it shares 25-30% identity with other members of the Coronavirus family including human coronaviruses 229E (HCoV-229E) and OC43 (HCoV-OC43). As HCoV-229E and HCoV-OC43 are ubiquitous in nature, the occurrence of false-positive reactions may be a result of cross-reaction amongst HCoVs. Thus, the potential cross-reactivity between HCoV N proteins was investigated by recombinant protein-based ELISA and Western blot assay.

3.3.1 Expression of recombinant SARS, OC43, and 229E CoV N proteins

The C terminal His tagged SARS-CoV, HCoV-229E and HCoV-OC43 N proteins were generated in insect cells using a baculovirus expression system. Protein expression was analyzed by Western blot analysis. A single band corresponding to SARS, and 229E nucleocapsid proteins were visualized at 55, and 56 kDa, respectively (Figure 17, lanes SARS-N and 229E-N, respectively). In contrast, OC43 nucleocapsid protein produced a band not only at 58 kDa, as expected, but also at 62 kDa (Figure 17, lane OC43-N). The production of a double band is most likely a result of cloning in frame with a mutated polyhedrin ATG (ATT) within the vector, which produces minor recombinant products that are larger than expected. Wild type baculovirus (AcMNPV) and uninfected cells were used as negative controls (Figure 17, lanes Wt and Cell, respectively).

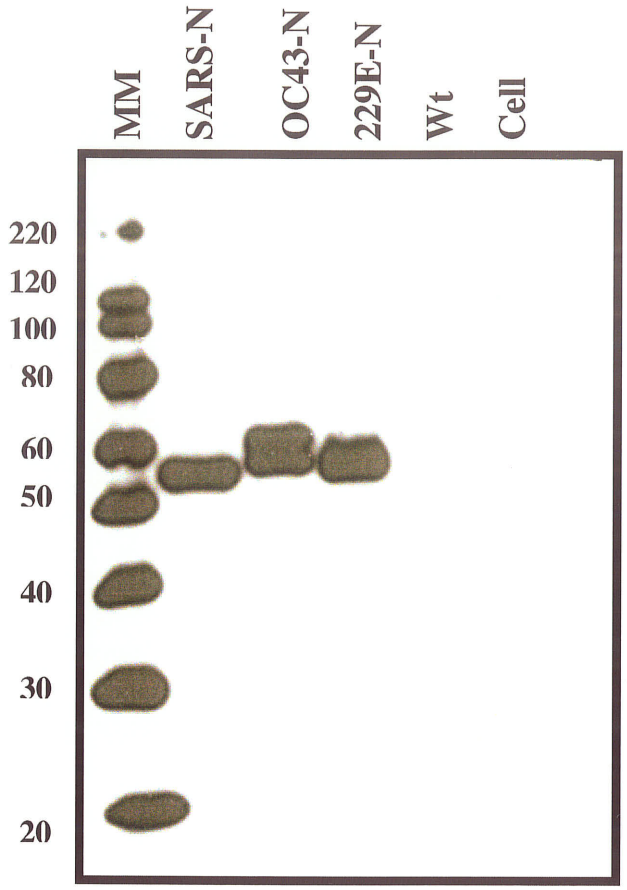

Figure 17. Expression of recombinant human CoV nucleocapsid proteins. Sf21 insect cells were infected with recombinant baculovirus expressing either the SARS-N (lane SARS-N, 55 kDa), OC43-N (lane OC43-N, 58 and 62 kDa), or 229E-N (lane 229E-N, 56 kDa). Proteins were detected with anti-His (1:2000) followed by goat anti-mouse HRP antibody (1:20,000). Lane MM, Magic Marker; Lane Wt, wild-type baculovirus (AcMNPV) infected cell control; Lane Cell, Sf21 cell control.

3.3.2 Investigate potential cross-reaction between antibodies against SARS, 229E, and OC43 CoV N proteins

Similar to the establishment of the baseline value for the specificity test, a panel of 190 sera from non-SARS infected individuals were used to generate the adjusted optical density (OD) values (OD of control – OD of wild-type infected cells) by HCoV N protein-based ELISA, thereby eliminating the non-specific responses to HCoV proteins.

The response to OC43-N, and 229E-N proteins were examined in serum samples recorded as SARS positive or negative by whole virus based ELISA and IFA (Table 5). Initially, 441 negative SARS sera were tested from 228 patients, of which 43.6%, and 68.3% of sera had antibodies against OC43-N, and 229E-N proteins, respectively. Upon testing positive SARS sera, 233 serum samples from 149 patients, the detection rate was 39.1% for OC43-N, and a surprising 83.3% for 229E-N. To determine if the increase in response to 229E-N was due to cross-reaction amongst HCoV antibodies, concurrent increases in antibodies to human CoV N proteins were evaluated in paired-patient serum samples, consisting of a SARS negative acute serum and SARS positive convalescent serum. Based on the analysis of paired SARS patient sera, of the 100 patients with both an acute and convalescent serum available for testing, 50% reacted only with SARS-N protein, while the remaining 50% showed cross-reactivity among antibodies against HCoV N proteins. More specifically, cross-reaction between antibodies against SARS-N and OC43-N, SARS-N and 229E-N, and lastly SARS-N, OC43-N and 229E-N, occurred in 3%, 44%, and 3%, respectively, of the tested patient sera.

The incidence of cross-reactivity between antibodies against nucleocapsid proteins of SARS-CoV and recognized human CoVs was further verified by Western blotting. Distinctive banding patterns were produced to SARS positive, negative, and non-SARS patient sera. In the majority of cases, the non-SARS sera bound to OC43-N or 229E-N proteins but not to SARS-N protein. However, on occasion, non-SARS sera bound to SARS-N, producing a false-positive reaction, as illustrated previously in Figure 16A (patient 1). Using paired samples, obtained from SARS patients with both acute (sera collected at a maximum day of 28 post illness onset) and convalescent-phase serum (sera collected beyond day 28 post illness onset), the presence and increase in antibodies against human CoV N proteins was tested. As expected, the majority (23/27) of the paired sera did not react to the SARS-N protein in the acute-phase but had a positive response in the convalescent-phase (Figure 18, patient 3). As well, patients who originally tested positive for OC43-N or 229E-N protein, showed no increase in antibody response to either proteins in the convalescent-phase serum (Figure 18, patient 4). In contrast, a few paired-patient sera displayed cross-reactivity amongst the CoV N proteins. In particular, several (7/27) convalescent-phase SARS serum samples showed increases in antibodies against 229E-N when compared with the corresponding acute-phase serum (Figure 19, patient 5 and 6). Wild-type baculovirus infected cells and uninfected Sf21 cells were used as negative controls, while Tor2 infected cells was used as a positive control (Figure 19, lanes Wt, Cell, and Tor2, respectively).

To prove that the reaction observed was in fact due to cross-reacting SARS-N antibodies and not by specific anti-229E or anti-OC43 antibodies, a competition assay

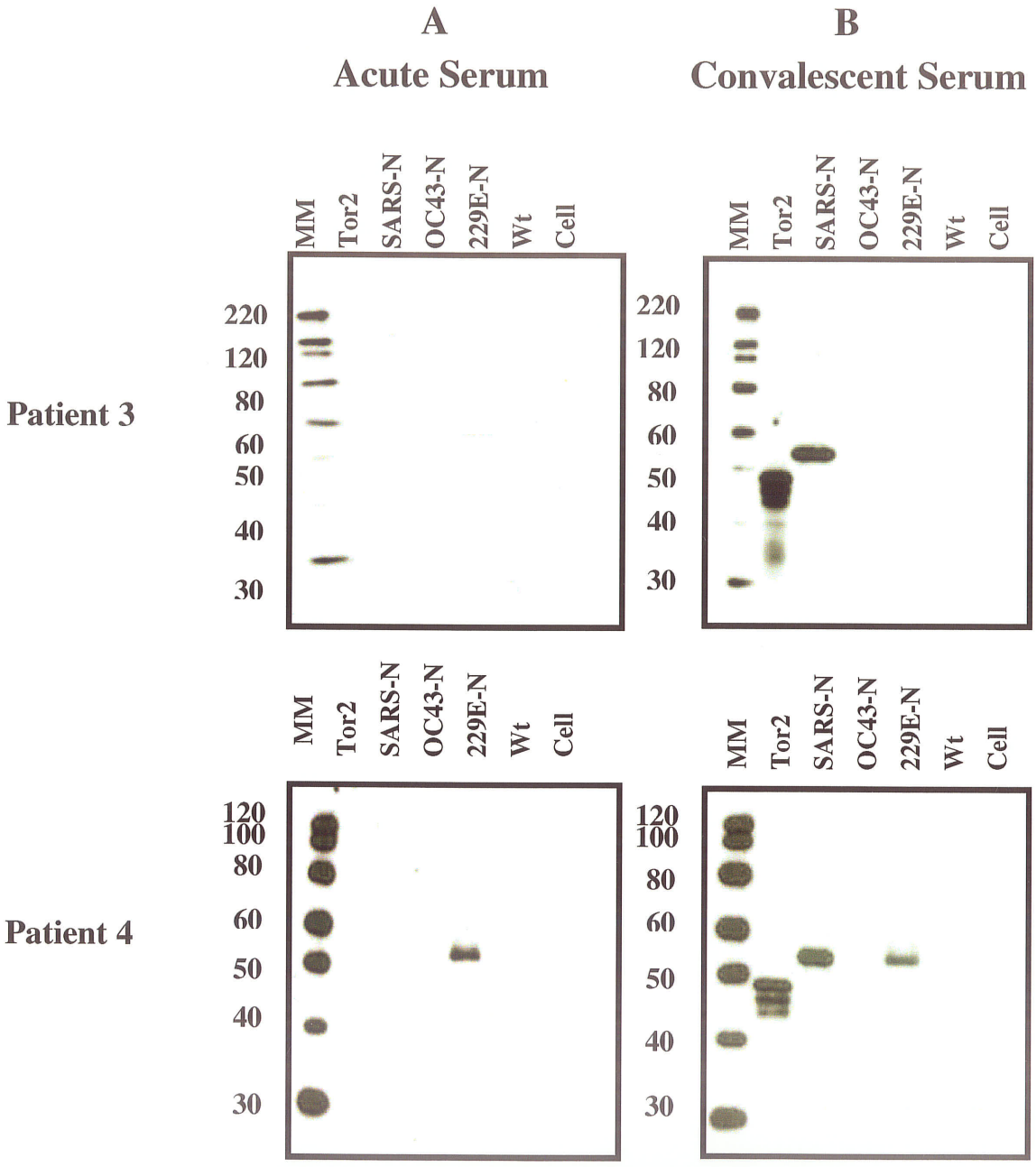

Figure 18. Typical immunoreactive patterns of the CoV nucleocapsid immunoblot with paired patient serum samples. Acute-phase (A), and convalescent-phase (B) sera (1:500) from patient 3 and 4 were detected by goat anti-human HRP antibody (1:30,000). Lane Tor2, Tor2 whole cell lysate control; Lane MM, Magic Marker; Lane Wt, Wt (AcMNPV) infected cell control; Lane Cell, uninfected Sf21 cell control.

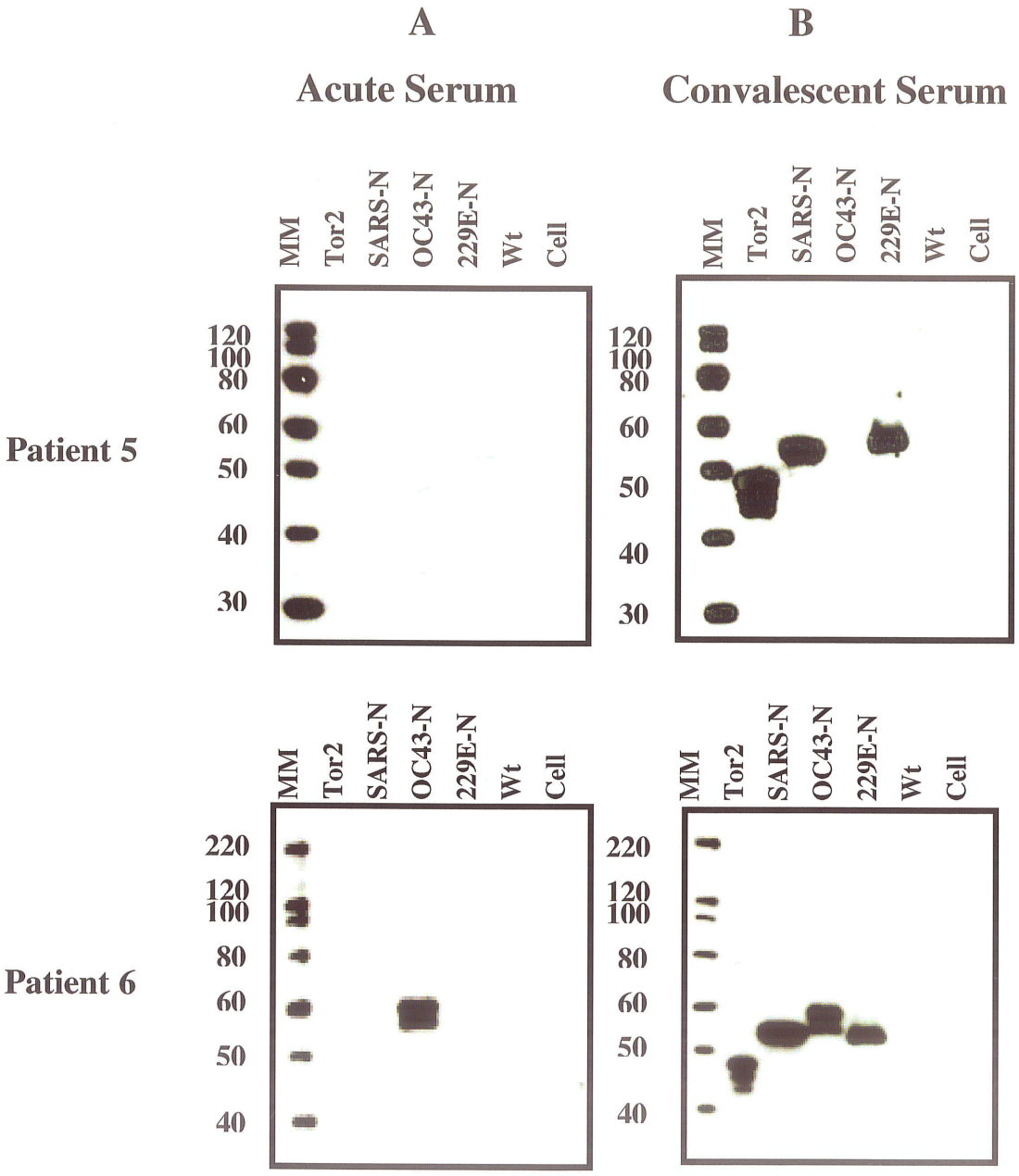

Figure 19. Immunoblot results illustrating cross-reactivity between SARS and 229E nucleocapsid proteins. Acute (A) and convalescent (B) paired-patient sera (1:500) from patient 5 and 6 were detected by HRP conjugated goat anti-human antibody (1:30,000). Lane Tor2, Tor2 whole cell lysate control; Lane MM, Magic Marker; Lane Wt, Wt (AcMNPV) infected cell control; Lane Cell, uninfected Sf21 cell control.

was performed. If the nucleocapsid proteins from SARS, OC43, or 229E could inhibit the binding of sera from SARS positive, negative, and non-SARS serum, cross-reaction amongst human CoV N antibodies is likely occurring. Immunoblot result of a representative seropositive response is shown in Figure 20A. Without competition, the patient's serum reacts with all of the human CoV nucleocapsid proteins. When pre-incubated with SARS-N, OC43-N or 229E-N protein there was a 52%, 100%, and 100% lower response in the respective band on the immunoblot. As the antigens competed solely with its corresponding N protein for binding to antibodies in the patient's sera, it appears as though each CoV N protein elicits a specific response in humans with no apparent cross-reaction between the three CoV N proteins. Alternatively, several patients displayed cross-reactivity amongst antibodies against SARS-N and 229E-N protein, as shown in Figure 20B. Without pre-incubation, the patient's serum reacts to both SARS-N and 229E-N. However, after pre-incubation with SARS-N not only was there a reduction in the intensity of the SARS-N band (71.8%), but also in the 229E-N band (52.4%). Similarly, pre-incubation with 229E-N resulted in a reduction in both SARS-N and 229E-N bands by 70.6% and 94.9%, respectively. Furthermore, a non-SARS patient serum that initially reacted to SARS-N in addition to the other two CoVs had an 83% to 100% lower response to SARS-N and 229E-N after incubation with either the SARS-N and 229E-N antigens (Figure 20C). No reaction was ever observed between the negative controls (wild-type baculovirus and Sf21 cells) and sera tested.

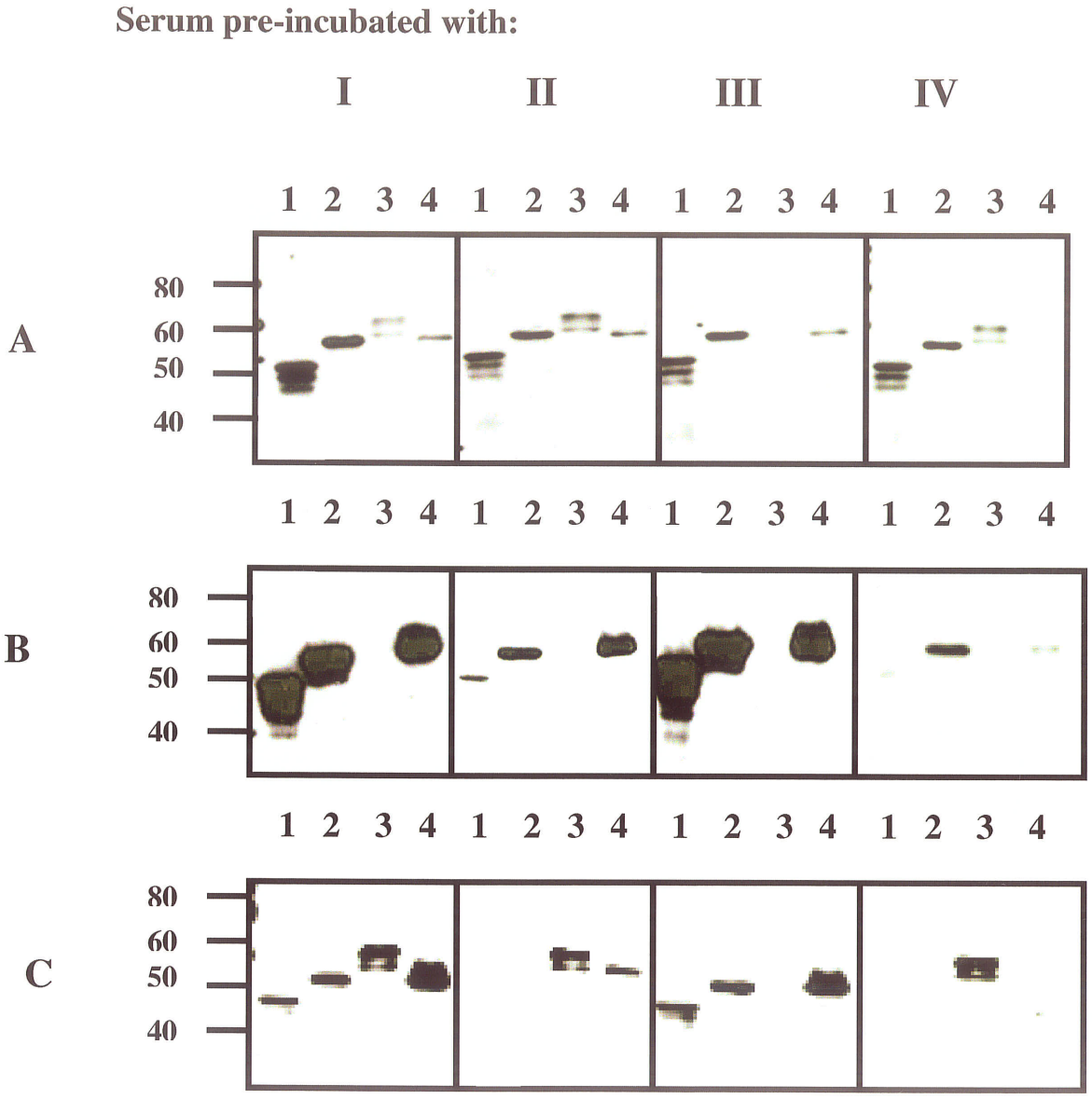

Figure 20. Immunoblot results of competition assay using full-length CoV N proteins. Representatives of a typical SARS positive serum (A), cross-reactive serum (B), and non-SARS serum showing a false-positive reaction (C) were pre-incubated with either: nothing (I), SARS-N (II), OC43-N (III), or 229E-N (IV) antigen. Lane 1, Tor2; Lane 2, SARS-N; Lane 3, OC43-N; Lane 4, 229E-N. No bands appeared on the Wt and cell control lanes (data not shown).

3.4 Important Regions within the SARS-CoV N Protein

Since the results, thus far, indicate that the SARS-N protein is the immunodominant protein of SARS-CoV and that anti-SARS-CoV N antibodies appear to cross-react with known human coronaviruses, truncated CoV N proteins were constructed to investigate the domains responsible for these findings.

3.4.1 Expression of truncated HCoV N protein constructs

The designs of the truncated coronavirus nucleocapsid fragments (Figure 9) were based on homologous regions between all three human CoVs (Appendix II). To eliminate cross-reaction amongst antibodies against HCoV, regions of similarity were removed. The constructs were amplified from the previously constructed recombinant plasmids containing either the full-length SARS, OC43, or 229E nucleocapsid genes. A start codon was added to the amino-terminal deletion mutants and the stop codon was removed from both the amino and carboxy-terminal deletion constructs such that the genes were in frame with the C-terminal His tag. The amplified products were then cloned into the eukaryotic expression vector, pBlueBac4.5, under the control of the polyhedron promoter. Recombinant viruses were produced as previously described. Protein expression of the truncated nucleocapsid His-tagged proteins was detected by immunoblot using anti-His antibody (1:2000) followed by an HRP-conjugated goat anti-mouse antibody (1:20,000). Immunoblot findings revealed bands corresponding to the N-terminal fragments of SARS-N-Nt (20 kDa), OC43-N-Nt (22 and 26 kDa), 229E-N-Nt (16 and 19 kDa) (Figure 21A), in addition to the C-terminal fragments of SARS-N-Ct (46 kDa), OC43-N-Ct (48 kDa), 229E-N-Ct (45 kDa) (Figure 21B). The double bands detected for N-terminal

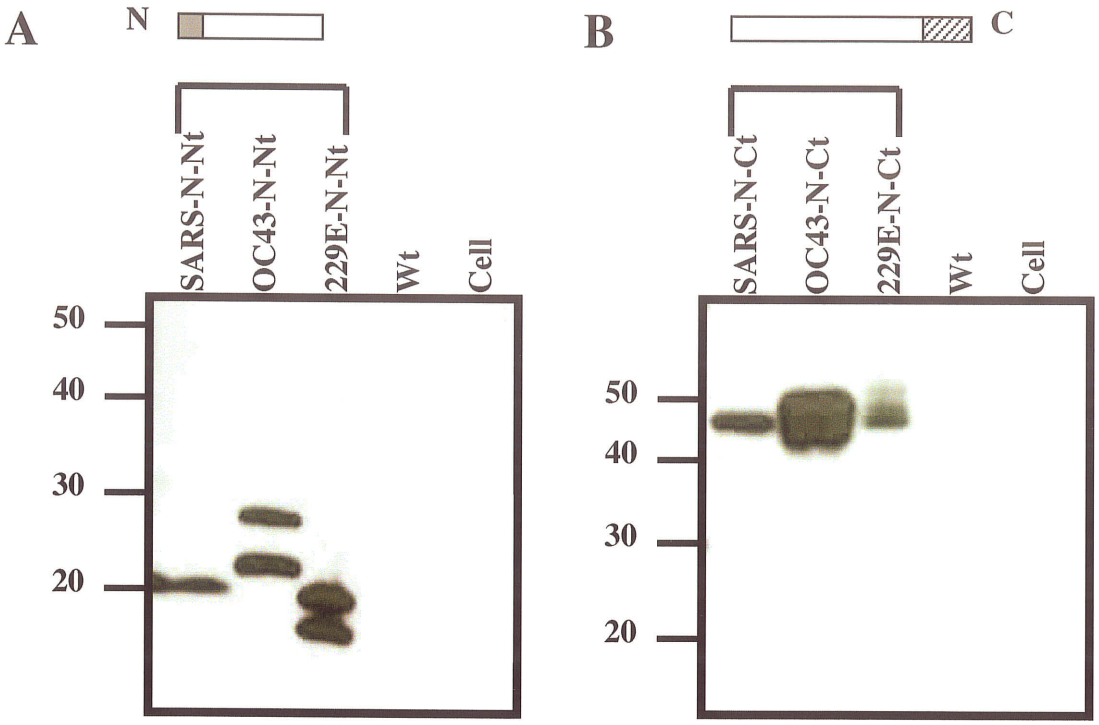

Figure 21. Expression of truncated HCoV N constructs confirmed by immunoblot. The N-terminal (A) and C-terminal (B) truncated HCoV N proteins were expressed in insect cells and detected by anti-His antibody (1:2,000) and anti-mouse HRP antibody (1:20,000). (A) N-terminal constructs: SARS-N (20 kDa), OC43-N (22 and 26 kDa), 229E-N (16 and 19 kDa); (B) C-terminal constructs: SARS-N (46 kDa), OC43-N (48 kDa), 229E-N (45 kDa). Lane Wt, Wt (AcMNPV) infected cell (negative control); Lane Cell, Sf21 cell control (negative control).

fragments of OC43-N-Nt and 229E-N-Nt are presumably due to an alternative initiation site within the vector. If translation does initiate at this site, it results in the production of minor recombinant products that are larger than expected, as previously noted. Wild type baculovirus (AcMNPV) and uninfected cells were used as negative controls (Figure 21, lane Wt and Cell, respectively).

3.4.2 Determination of significant domains within the SARS-CoV N protein

To determine the validity of the truncated CoV constructs, HCoV-229E, HCoV-OC43 and SARS-CoV positive serum samples were reacted against the N and C-terminal CoV nucleocapsid constructs. After reacting the N and C-terminal nucleocapsid constructs of 229E and OC43 against human sera known to be HCoV-229E and HCoV-OC43 positive, the resulting immunoblots were negative for all constructs (data not shown). Similarly, the N-terminal fragments of SARS-N did not react with any of the sera tested (data not shown). In contrast, the C-terminal fragment of SARS-CoV N protein from SARS positive patients exhibited a strong response towards the N protein fragment derived from the C-terminus of the SARS-CoV nucleocapsid protein (Figure 22, patient 5). Therefore, further studies were focused on the reactivity of the C-terminal SARS-N construct to investigate the regions responsible for immunodominance and cross-reactivity amongst antibodies against human CoV N proteins. Numerous sera (17/31) from SARS positive patients exhibited a strong response towards the N protein fragment derived from the C-terminus of the SARS-CoV nucleocapsid protein. Moreover a few sera that previously displayed false-positives no longer reacted with SARS-CoV N protein (Figure 22, patient 1). This data indicates that the C-terminus of the SARS-N

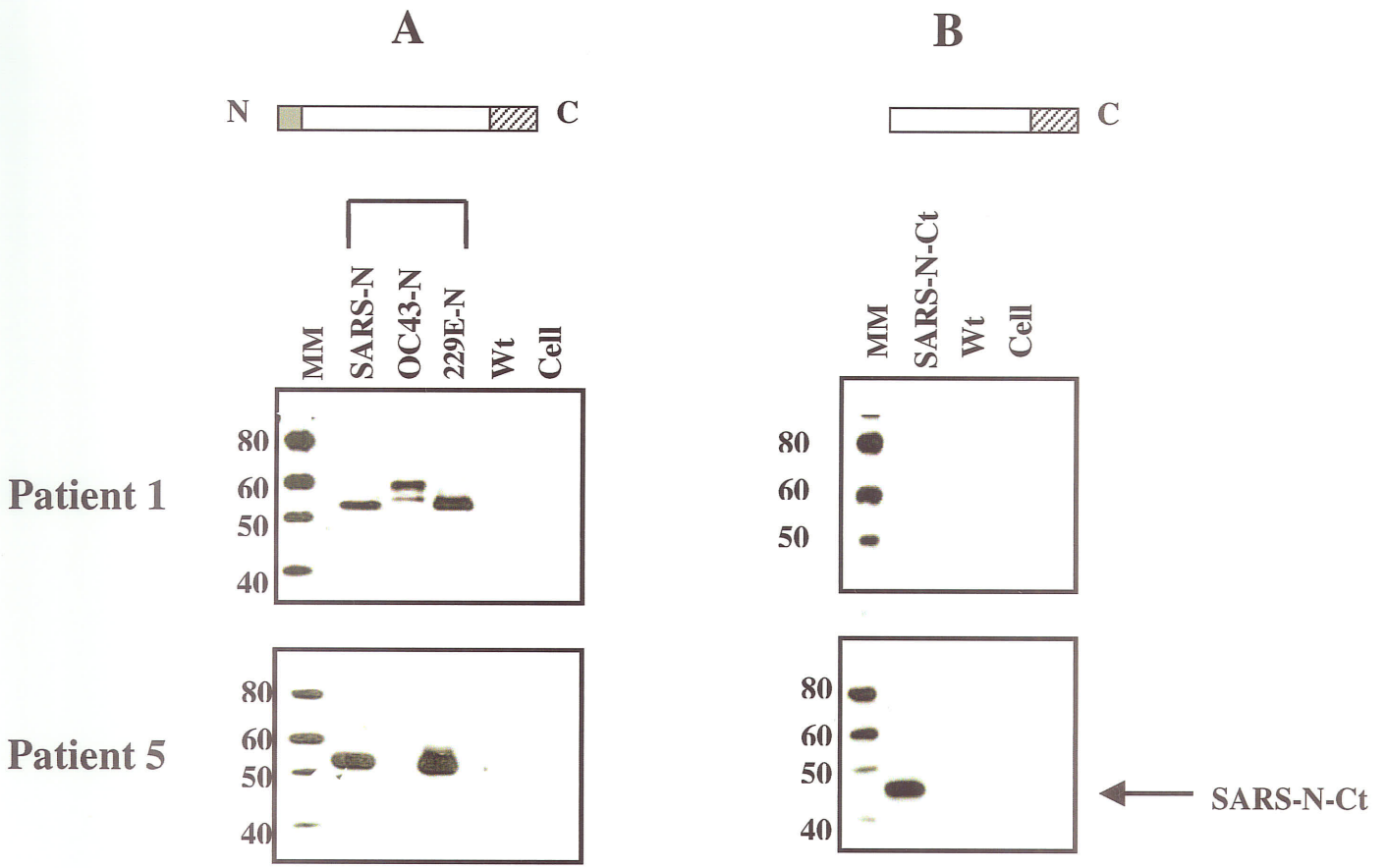

Figure 22. Immunoblot of full-length and truncated HCoV nucleocapsid constructs against human sera. Representative sera from seropositive non-SARS patient (patient 1), and SARS positive patient (patient 5) reacted with full-length (A), and C-terminal (B) HCoV N protein constructs. Lane MM, Magic Marker; Lane Wt, Wt (AcMNPV) infected cell control; Lane Cell, Sf21 uninfected cell control.

protein is an immunodominant region that seems to be specifically recognized by anti-SARS-CoV N antibodies.

To further explore these findings, we tested a larger population of SARS positive, negative and non-SARS sera against the C-terminal fragment of SARS-N, by ELISA (Table 6). Amongst the 233 positive SARS sera tested from 149 patients, the reaction rate was 96.6% against the C-terminal construct of SARS-N. Upon analyzing 441 negative SARS serum from 228 patients, 5.9% reacted to the C-terminal region of SARS-N. Lastly, 1.0% of the non-SARS patients elicited a response to the C-terminal constructs of SARS-N.

Comparison of the positive responses to full-length and truncated HCoV N constructs in SARS positive, negative and non-SARS sera is depicted in Figure 23. Clearly, the response to the truncated SARS-N constructs in the three groups of sera remained virtually identical to the response generated by the full-length protein. As an alternative, competition assays were performed to determine whether the C-terminal nucleocapsid constructs of OC43 or 229E could inhibit the binding of human sera to the C-terminal nucleocapsid construct of SARS. A typical immunoblot response is shown in Figure 24A, in which the C-terminal constructs of OC43-N or 229E-N did not inhibit the binding of human sera to the C-terminal SARS-N construct. Upon testing a false-positive non-SARS serum, the C-terminal construct of 229E-N protein partially inhibited the binding of human sera to the C-terminal construct of SARS-N (Figure 24B). This indicates that cross-reaction is still occurring amongst the HCoVs and that the false-positive responses could be, in part, attributed to this phenomenon.

Table 6. Summary of ELISA results investigating the response to truncated HCoVs nucleocapsid proteins in SARS positive, negative, and non-SARS sera

Sera	C-terminal Construct	No Positives	Total No.	% Positive
SARS POSITIVE SERA	SARS-N-Ct	225	233	96.6
SARS NEGATIVE SERA		26	441	5.9
NON-SARS SERA		2	192	1.0

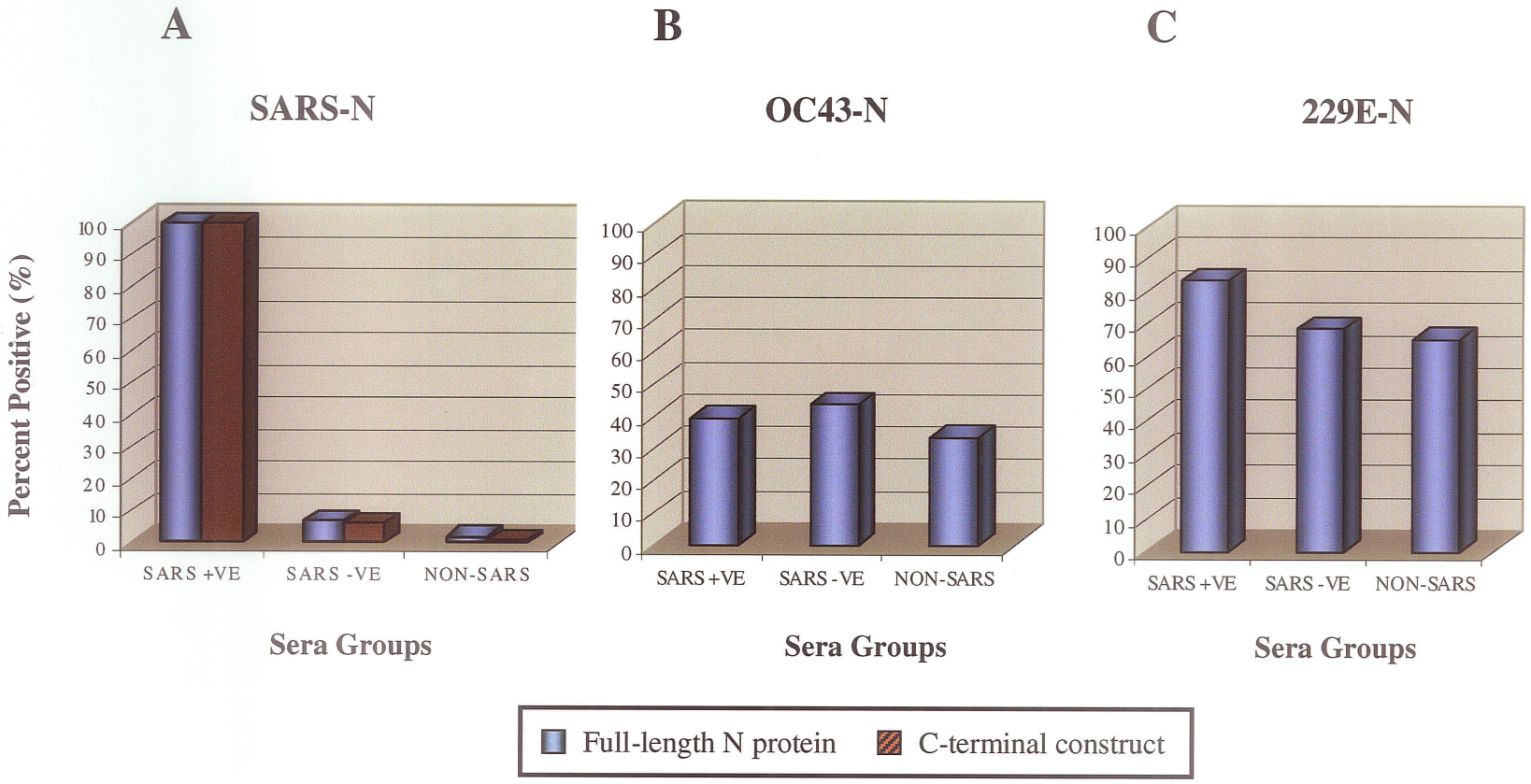

Figure 23. Comparison of full-length and truncated HCoV nucleocapsid construct detection rates in human sera by ELISA. Shown are the positive responses of SARS positive, negative, and non-SARS sera.to full-length (blue bars) and C-terminal construct (purple bars) of SARS-N (A), OC43-N (B) and 229E-N (C).

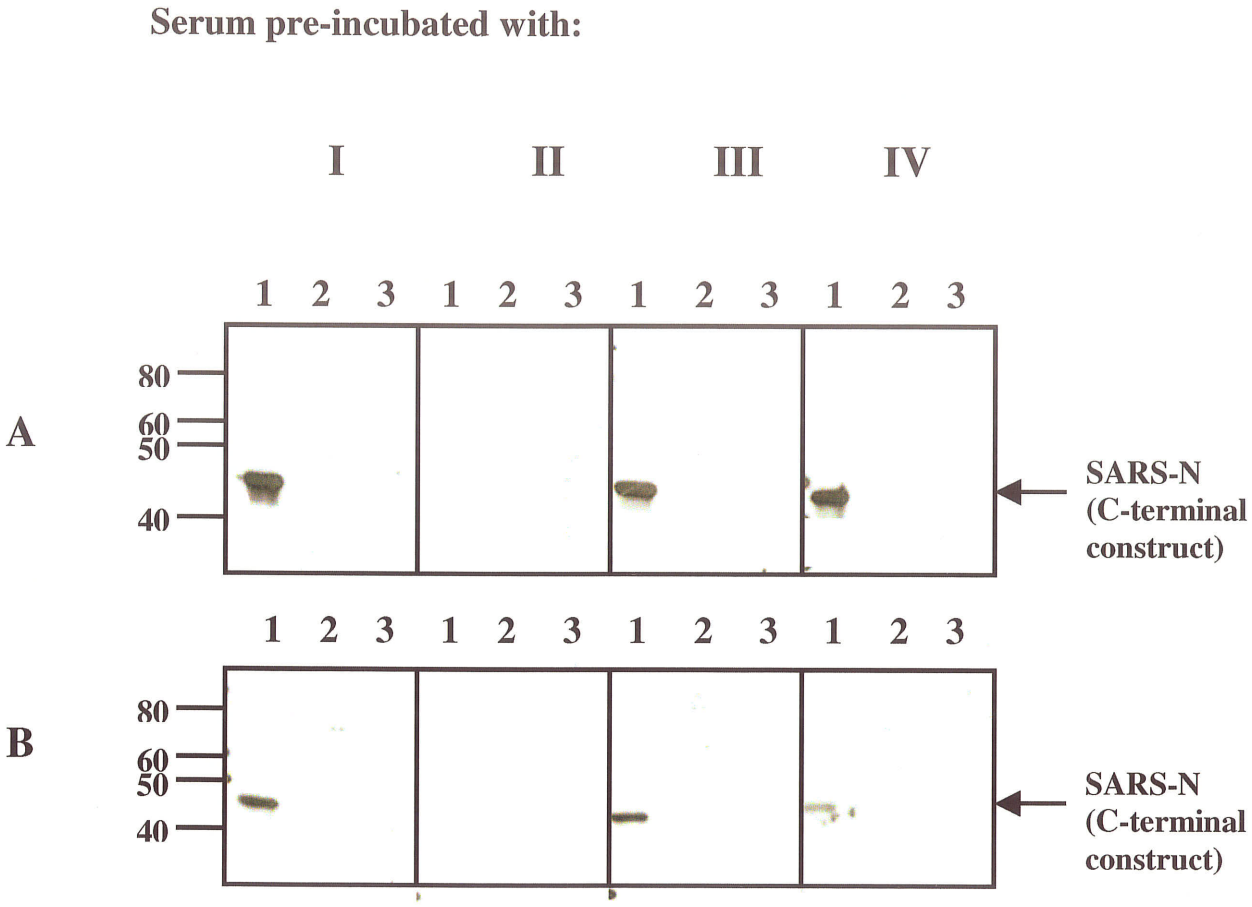

Figure 24. Immunoblot results of competition assay using truncated CoV N constructs. Typical response of a SARS positive (A), and seropositive non-SARS serum (B) when pre-incubated with either: nothing (I), or the C-terminal construct of SARS-N (II), OC43-N (III), or 229E-N (IV) antigen. Lane 1, SARS-N (C-terminus); Lane 2, OC43-N (C-terminus); Lane 3, 229E-N (C-terminus). No bands appeared on the Wt and cell control lanes (data not shown).

4 DISCUSSION

4.1 Exploring the human immune response to SARS-CoV infection and other known HCoVs

The newly emerged SARS is a serious respiratory illness of global significance. Due to its high mortality rate, and highly contagious and acute nature it imposes a tremendous psychological and economic burden on public health. Yet, many aspects of the disease and its etiological agent, SARS-CoV, have yet to be fully understood. Thus, the present study was established to provide insight into the human antibody response to SARS-CoV infection, in addition to assessing the potential antibody cross-reactivity between human coronaviruses. Taken together, the crucial information gained could be used for the development of therapeutic strategies and diagnostic tools.

To investigate the presence of antibodies to human coronaviruses and further profile the humoral response to SARS-CoV infection, two fundamental methods were employed: ELISA and Western blot analysis. Both assays were based on recombinant CoV proteins. Although IFA, which detects antibodies to antigens present in virus-infected cells, is considered the "gold-standard" antibody test, it does not reveal which viral antigens are targeted by the immune response. Similarly, whole-virus based ELISA does not specify which viral proteins are responsible for generating an immune response. Knowing the viral targets is important for several reasons. First, this allows the development of cell-free antibody tests that are less cumbersome and subjective than IFA and that can be used for mass screening in times of epidemics. Second, this enhances our understanding of the immunopathology of the disease, and moreover, helps in design of a

vaccine. In the present study, we identify the viral antigens to which the patients with SARS had responded and profile SARS-CoV specific antibodies using recombinant protein-based Western blot and ELISA assay. We further employ these assays to assess immunoreactive domains and the potential serological cross-reactiviton amongst human coronaviruses.

As a foundation for the study, we evaluated serum samples from probable and suspected SARS patients collected throughout Canada. The serum samples covered collection days from day 1 to day 256-post onset of illness, allowing an extensive look into the antibody response to SARS-CoV at different stages of disease progression.

4.2 Analysis of immunoreactive SARS-CoV structural proteins

To characterize the host specific immunity against SARS-CoV antigens, the SARS-CoV structural proteins, N, S, M, and E, were cloned into a eukaryotic expression vector and expressed in Sf21 insect cells. On the basis of genomic data, the expected sizes of the structural proteins (including the 6xHis tag) were: 142 kDa (S protein), 50 kDa (N protein), 28 kDa (M protein), and 12 kDa (E protein). Although the theoretical value of the S protein is smaller than the expressed band, it is glycosylated during infection of host cells (Gallgher *et al.*, 2001; Krokhin *et al.,* 2003). Therefore, it is not surprising that the SARS-CoV S protein has a significantly higher molecular mass than the theoretical value. Comparable occurrences have been observed by Ren *et al.* (2003) and Huang *et al.* (2004).

Although members of coronavirus family have similar structural proteins, the antigenicities and roles of these viral proteins in immunity vary. For instance, the IBV

spike glycoprotein is more antigenic than the N, M, or E proteins (Ignjatovic *et al.,* 1993), whereas the M protein of canine coronavirus has consistently been detected in seropositive dogs (Elia *et al.,* 2003). In addition, the N protein is a strong immunogen in numerous coronaviruses, including murine CoV (Wege *et al.,* 1993), turkey CoV (Akin *et al.,* 2001), and porcine reproductive and respiratory syndrome CoV (Casal *et al.,* 1998). In support of the latter, we found that human sera generated a strong immune response to the N protein at all stages of SARS-CoV infection (Table 4, Figure 11). By day 1 after disease onset, not only is the N protein detectable in SARS patient serum samples (Che *et al.,* 2004), but the results indicate antibodies against the N protein are also present at this time. The existence of N protein and antibodies against it in the early acute-phase SARS sera suggests that the N protein of SARS-CoV may be released, as a major viral immunogen, from the virus or infected cells into the blood at an early stage of infection. Moreover, samples from convalescent patients were 100% positive for IgG antibodies against N and remained so even at day 256-post onset of illness when tested by ELISA. This is consistent with several independent studies in which antibodies against the N protein are detected in >90% of sera obtained from convalescent SARS patients (Shi *et al.,* 2003; Wang *et al.*, 2003; Guo *et al.,* 2004; Leung *et al.,* 2004; Tan *et al.,* 2004;) and found to persist in SARS-CoV patients for >13 weeks after the onset of illness (Li *et al.,* 2003). In a recent study, the SARS-CoV N protein is capable of generating strong N-specific humoral and cellular immune responses (Zhu *et al.,* 2004), which might potentially be useful in the control of infection by SARS-CoV. Collectively this data suggests that the N protein plays a significant role in the generation of the immune response against SARS-CoV infection.

Interestingly, unlike some coronaviruses in which the M protein is the most abundant (Siddell *et al.,* 1995), the N protein appears to be the major protein produced in SARS-CoV infected cells. This was evident upon Western blot analysis, as the predominant antigen in the crude viral extract of Tor2 was the N protein. In addition, we found evidence that the N antigen is degraded in cell cultures of the virus by the presence of multiple bands. These findings have been observed in numerous coronaviruses, and are likely a result of cleavage by host cell caspases that are activated during CoV infection (Eleouet *et al.,* 2000; Leung *et al.,* 2000).

Aside from the N protein, the SARS-CoV S protein was the most frequently targeted antigen by the immune response of patients with SARS (Table 4, Figure 11). Similar results have been documented by Huang *et al.* (2004) and Leung *et al.* (2004). By recombinant protein-based ELISA, antibodies against the spike protein were detected as early as day 3 after disease onset. Despite its early appearance, it appears to play a greater role later in infection, as the detection rate of convalescent-phase sera (76%) was greater than that of the acute-phase sera (67%). Furthermore, by day 122 post symptom onset, all SARS patients showed immunoreactivity to the spike protein. Comparable findings were observed by Western blot (Figure 12B). Previous studies of known coronaviruses have recognized the S protein to be responsible for inducing host immune responses and virus neutralizing antibodies (Callebaut *et al.,* 1996; Gomez *et al.,* 1998; Holmes et al., 2003). This phenomenon is not unforeseen, as the unique projection of the spike protein makes it the earliest recognized antigenic epitope by the host immune system compared to other structural proteins (Callebaut *et al.,* 1996; Homberger *et al.,* 1996; Sanchez *et al.,* 1999). With regard to SARS-CoV, it has been found that the S

protein can induce a T cell response in addition to a neutralizing antibody response (Navas-Martin *et al.,* 2003; Yang *et al.,* 2004). Therefore, antibodies against S may play an important role in controlling and clearing SARS-CoV infection.

The remaining SARS-CoV structural proteins, M and E, appear to be minor targets of the immune response. Both recombinant proteins displayed little immunoreactivity to any of the human serum samples in the study when tested by ELISA (Table 4, Figure 11). A similar behavior was observed by Western blot performed with human sera, as a band <50 kDa was seldom immunostained. This further indicates that the M and E proteins do not elicit antibody production or generate very low antibody titers in SARS patients. These findings are consistent with several independent studies, which report that antibodies against M and E proteins are generally low or not present in SARS patient sera (Wang *et al.,* 2003; Guo *et al.,* 2004, Leung *et al.,* 2004. Tan *et al.,* 2004). Despite these findings, M and E are important for viral assembly as together, they are sufficient for the formation of virus-like particles in insect cells (Ho *et al.,* 2004).

It is important to note that the above results are based on multiple patient sera. Although individual patient serum samples show similar trends in regards to the immune response to each antigen, this is not reflected when the sera are combined. Rather, the highly variable detection rates of SARS-CoV proteins from one individual to another on the same day (Figure 11) may be a result of individual biological variation in the immune system. It has been reported that the mean time to seroconversion for SARS-CoV infection is 20 days (Peiris *et al.*, 2003); however, some patients have a delayed immunological response and may not seroconvert until week 6 (He *et al.,* 2004). Our findings support the latter as the mean time to seroconversion was found to be 43.4 days.

Furthermore, the health and treatment received for each patient is unknown. Therefore, these factors could be affecting the appearance and strength of antibody responses to SARS-CoV antigens.

4.3 Analysis of SARS-CoV nucleocapsid specific antibodies

Since SARS-CoV N protein appears to be the main target of the immune system, further studies were aimed at profiling the specific anti-nucleocapsid antibody response during SARS-CoV infection. Utilizing the recombinant SARS-CoV N construct, the IgG, IgM, and IgA antibody responses were investigated from day 2 to 256 after the onset of illness in serially collected SARS serum samples by ELISA and Western blot analysis.

Previous profiles of antibodies against SARS-CoV N protein have generated conflicting results. Li *et al.* (2003) reported a lag period after SARS infection in which no humoral response to SARS-CoV was detectable until the second week of illness, whereas Woo *et al.* (2004) found detectable levels of IgG, IgM, and IgA only by the third week. In the present study, the levels of the three antibodies increased to detectable levels as early as week 1 by recombinant N protein-based ELISA and Western blot assay. Although a few patients generated IgM and IgA antibodies slightly earlier than that of IgG, the majority of patients had an advanced and much greater IgG response throughout the course of disease than either IgM or IgA (Figure 13). Theoretically, IgM should be the first antibody produced if these patients had a true primary response to SARS, and should be accelerated if it was a secondary response (Hsueh *et al.,* 2004). One possible explanation for our findings is that IgM antibody to SARS-CoV persisted for a short time and had disappeared before the patients' sera were collected, or, some patients were

infected with SARS-CoV before the documented infection during the epidemic. Although this is difficult to exclude, it is extremely unlikely as sera collected prior to the SARS outbreak showed little recognition to SARS-CoV. Alternatively, the weak signal of IgM might have resulted from the characteristics of IgM itself, since the affinity of IgM to antigens is relatively low as compared with that of IgG or IgA (Wu *et al.,* 2004). Thus, our findings could reflect the differential sensitivity of ELISA assay for the detection of IgM or IgA rather than the biology of the host response.

Over the course of illness, the anti-nucleocapsid IgM response produced a steady decrease in detection (Figure 13). In fact, the average IgM antibody titer nearly dropped below detectable levels between days 94 to 122-post illness onset. These results are similar to a previous study that found the presence of low levels of IgM and IgA at 100 days after the onset of illness (Hsueh *et al.,* 2004). Despite the diminishing response, anti-nucleocapsid IgM was still detectable in patient sera by day 198 post onset of illness. Of note, IgM experienced higher detection rates in acute-phase sera (56%) than in convalescent-phase sera (40%); however, the opposite was true for the mean IgM titer in SARS patient sera, which reached a mean titer of 1:830 in acute-phase sera and 1:930 in convalescent-phase sera (Figure 14). The peak mean titer was attained slightly after reaching the maximum detection rate of IgM (days 43 to 49, and days 36 to 42, respectively). Although detectable levels of IgM were evident as early as day 1 after symptom onset, Li *et al.* (2003) reported that patients were positive for IgM only from week 2 of illness when assayed with coarse viral lysates. Thus, the use of recombinant N protein may increase the sensitivity of ELISA assays and allow for earlier detection of SARS-CoV infection.

With respect to anti-nucleocapsid IgA response, there was a moderately fluctuating response in human sera throughout the course of disease, as detected by ELISA (Figure 13). In terms of reactivity, we found that specific IgA antibodies already existed 1 day after illness onset in some cases. Further investigation of the results revealed that the titer of IgA were greater in acute-phase sera (1:1327) when compared with that of the convalescent-phase (1:977), while the positive detection rate remained nearly identical in acute and convalescent-phase sera (~74%) (Figure 14 and 13, respectively). In response to infection of mucosal surfaces, secretory IgA antibodies are produced as an important defense mechanism against invasion of deeper tissues by these pathogens (van Ginkel *et al.,* 2000). Since the human respiratory tract is composed entirely of mucosal surfaces and proposed to be the major site of SARS-CoV infection in humans, the synthesis of specific IgA antibodies in the early stage of a SARS-CoV infection is reasonable, and has been observed in other related coronavirus studies (de Arriba *et al.,* 2002; Loa *et al.,* 2002; Naslund *et al.,* 2002).

Finally, a look into the anti-nucleocapsid IgG response revealed a peak IgG antibody value around 60 days after onset of obvious symptoms (Nie *et al.,* 2003). Correspondingly, the present study found that the median IgG titer had a gradual and erratic rise, to a peak value of 1:3200 at days 94 to 122 post symptom onset (Figure 14). Contrasting the varying titer, the level of anti-nucleocapsid IgG positive patients remained consistently high throughout the course of disease and was still detectable at day 256 post onset of illness (Figure 13). The prolonged IgG production suggests that not only does IgG play a principal role in the humoral immune response against acute SARS-CoV infection, but it may also exert a crucial action in cleaning up the residue of virus

foci in the recovery phase (Chen *et al.,* 2004). It has been noted that IgG antibody to SARS-CoV are associated with host T cell immunity (Chen *et al.,* 2004). Therefore, we speculate that IgG antibodies against N may contribute to the humoral immune response by protecting patients against SARS, as has been observed for other coronaviruses (Siddell, 1995).

4.4 Major immunodominant domain of SARS-CoV N protein

Although many factors influence antigen-antibody interactions, two major features, charge-charge interactions and hydrogen bonding caused by hydrophilicity, have been hypothesized to be crucial for an epitopic site (Hopp *et al.,* 2003). According to hydrophobicity analysis, most regions of the S and M protein of SARS-CoV are very hydrophobic with fewer polar amino acids than the N protein (Wang *et al.,* 2003). This supports our previous findings in which the S and M protein generated a lower immune response than the N protein. When compared with the NH_2 terminus, the COOH terminus of N protein is a region of relatively low hydrophobicity and has numerous polar amino acids, which make it an excellent antigenic region (Wang *et al.,* 2003). In support, removal of the C-terminal region from all human CoV N proteins resulted in complete abolishment of reaction with human sera. Conversely, truncation of the N-terminal region of SARS-CoV N protein did not alter its ability to detect SARS positive patients, as the reaction rate of the C-terminal construct was nearly identical to that of the full-length SARS-CoV construct (Figure 23). Combined, these results indicate that the most immunoreactive epitopic site in the SARS-CoV is located at the COOH terminus of the N protein.

Numerous studies, analyzing the antigenicity of different regions of the SARS-CoV N protein, have had similar findings (Figure 25). Tan *et al.* (2004) found that the N-terminal region of the N protein is dispensable, as all tested sera showed immunoreactivity towards the truncated protein spanning amino acids 120 to 422. He, Q. *et al.* (2004) further elucidated these results by identifying a truncated 195 amino acid fragment (N195), derived from the C-terminus of the nucleocapsid protein, which reacted with all patient sera in the later stage of SARS infection. Confirming these results, Chen *et al.* (2004) discovered four important regions within the nucleocapsid protein, designated EP1 (aa 51-71), EP2 (aa 134-208), EP3 (aa 249-273) and EP4 (aa 349-422), the last two of which fall within the 195 truncated fragment identified by He, Q. *et al.* (2004). Using SARS sera to screen a set of overlapping peptides that span the N protein, He, Y. *et al.* (2004) found two immunodominant regions that lie within fragments EP2 and EP4. Lastly, Wang *et al.* (2003) analyzed epitopes of the N protein using synthetic peptides and found N66 and N371 (aa spanning 66-67, and 371-404, respectively) to be important epitopes. Notably peptides encompassing amino acids 371-390, and 385-407, located at the COOH terminus, inhibited the binding of antibodies to SARS-CoV lysate and had the highest reactivity against SARS sera, binding to antibodies in >94% of SARS sera (Wang *et al.,* 2003). Collectively, these findings indicate that the COOH terminus of the N protein contains the strongest immunogenic epitopes, which could play a crucial role in diagnosis of SARS-CoV infection.

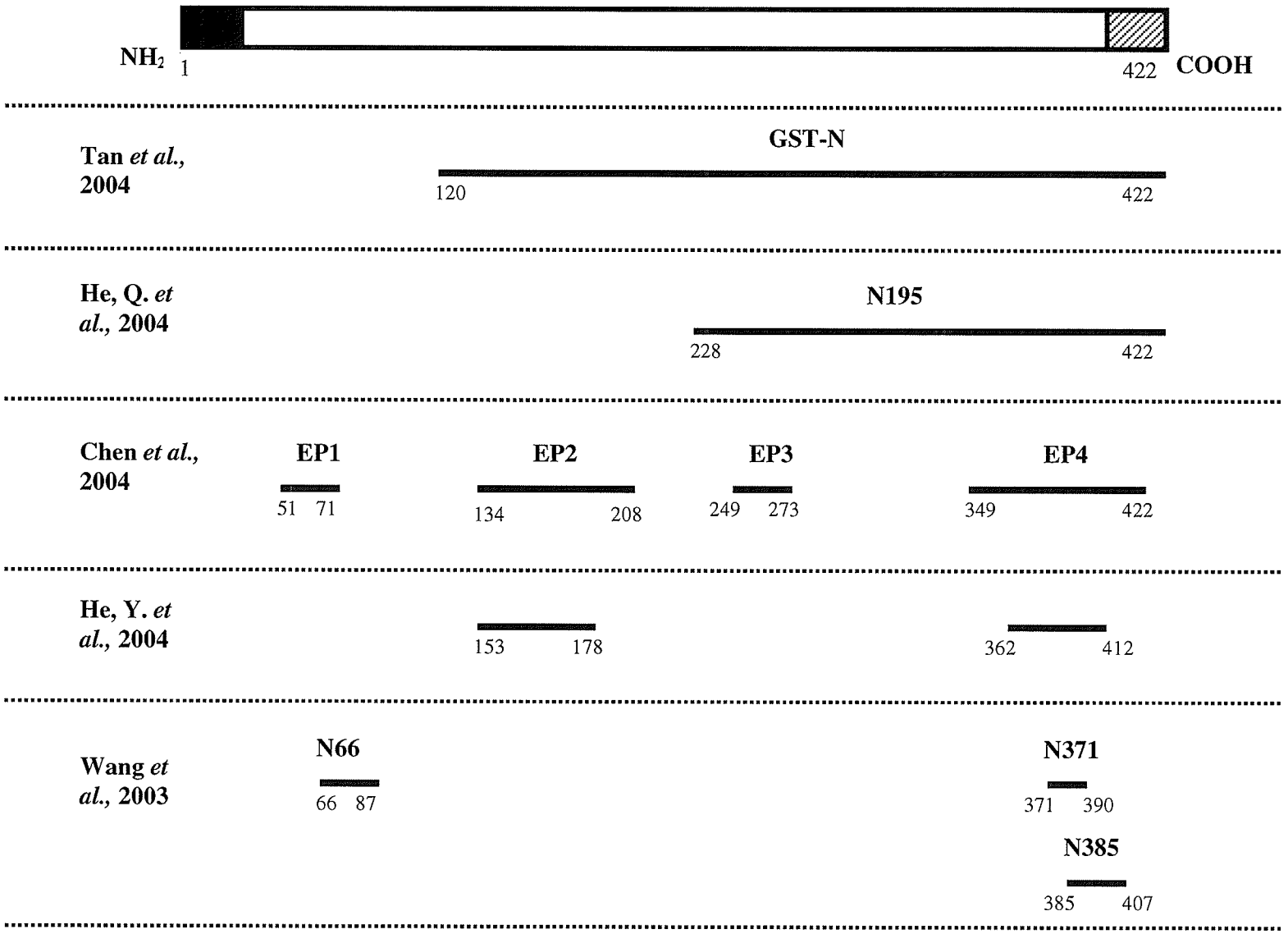

Figure 25. SARS-CoV N protein epitope research.

4.5 Specificity amongst human coronaviruses

With the identification of a possible animal reservoir of SARS-CoV there is a continual threat of re-emergence. Rapid and accurate diagnosis is critical for providing optimum patient care and in helping contain disease spread. From the experimental results, SARS-CoV N shows promise for diagnosis of SARS-CoV infection. Not only is it abundantly expressed, the N protein is also highly conserved among strains (Ruan *et al.,* 2003). Notably, early-phase sera displayed immunoreactivity to the N protein, an essential attribute for early diagnosis of infection. In addition to these features, a good diagnostic tool is not only sensitive but specific as well. Thus, we investigated the potential cross-reactivity between HCoV N proteins by recombinant protein-based ELISA and Western blot assay.

Initially, recombinant nucleocapsid proteins of HCoV-OC43, HCoV-229E, and SARS-CoV were used to screen SARS negative and non-SARS sera (Figure 16). The first indication that cross-reaction may be occurring between antibodies against HCoVs was the appearance of unexpected seropositive responses from SARS negative sera and non-SARS sera that were collected at least one year prior to the SARS outbreak. By recombinant-protein based ELISA, a surprising 6.6% of SARS negative and 2.1% of non-SARS sera produced a positive response to SARS-CoV N (Table 5). Similar results were detected by Western blot assay, using recombinant CoV N proteins, as shown in Figure 16. Although these results are concerning, they are not unlike recent studies, which have detected a small proportion of seropositive responses in healthy controls tested for antibodies to SARS-CoV by ELISA (Guan *et al.,* 2004; Huang *et al.,* 2004; Woo *et al.,* 2004).

Upon further examination of the response of non-SARS, negative and positive SARS sera to OC43 and 229E nucleocapsid proteins, we found that a large proportion of patients had a positive response to both CoVs, as detected by ELISA (Table 5) and Western blot (Figure 18, patient 4; Figure 19, patient 6). This is not surprising, as seroprevalence surveys have shown that 86% to 100% of all adults have serum antibodies to HCoV-OC43 and HCoV-229E (Macnaughton, 1982; Cereda *et al.,* 1986; Schmidt *et al.,* 1986; Hruskova *et al.,* 1990). Additional analysis revealed that all three groups of sera had a comparable reaction to OC43-N protein, whereas the response to 229E-N protein was much greater in SARS positive sera than either SARS negative or non-SARS sera (Table 5). To clarify these results we analyzed paired-patient sera in search of significant antibody increases from acute to convalescent-phase serum. We found that an increase in detection of antibodies against 229E-N was frequently accompanied by an increase in antibodies against SARS-N. Likewise, Western blot analysis of convalescent patient serum revealed a positive response not only to SARS-N but also to 229E-N, despite the fact that the corresponding acute-phase patient serum was negative for both (Figure 19). One possible rationale for this occurrence is co-infection of SARS-CoV and HCoV-229E. However, the probability of this occurring at such a high frequency is unlikely. Instead, parallel increases in anti-229E-N and anti-SARS-N antibodies could be due to cross-reactivity between the anti-nucleocapsid antibodies of SARS-CoV and the other known HCoVs. In support of this hypothesis, cross-reaction was evident between the antibodies against the nucleocapsid proteins of SARS-N and 229E-N by competition assay (Figure 20B and 20C).

Although cross-reaction between antibodies against HCoVs may partially explain the detection of false positives and the parallel increase in antibodies to SARS and 229E N proteins, it cannot account for all cases. Thus, as an alternative our findings could be a reflection of the phenomenon of "original antigenic sin". First observed in influenza virus infections, it is the tendency of humans to preferentially generate antibody responses to an original virus elicited by infection of an antigenically related virus (Deutsch *et al.,* 1973; East *et al.,* 1980; Inouye *et al.,* 1984; Anderson *et al.,* 2001;). Thus, if a patient had been exposed to HCoV-229E prior to SARS-CoV infection, it is possible that the subsequent SARS-CoV infection causes a selective production of antibodies, which are common to both the priming and boosting viruses. This has been observed by East *et al.,* (1980) who reported that antibodies produced in response to the boosting myoglobin are almost exclusively directed towards antigenic regions common to the priming and boosting myoglobins. This could account for the concurrent increase in antibodies to SARS-N and 229E-N in convalescent-phase patient's, as well as explaining the occurrence of false-positive responses.

The results from the present study suggest that antibodies against SARS-CoV and HCoV-229E (group I) cross-react more frequently than antibodies against SARS-CoV and HCoV-OC43 (group II) based on recombinant SARS-CoV nucleocapsid protein-based ELISA, immunoblot analysis, and competition assay. However, these results challenge the current knowledge of amino acid identity between CoVs, as SARS-CoV shares greater homology with HCoV-OC43 (32.7%), a group II CoV, than with HCoV-229E (21.3%), a group I CoV. Analogous to our findings, Ksiazek *et al.* (2003), have shown that SARS-CoV-infected Vero cells react with polyclonal antibodies from

antigenic group I CoVs, including human covornavirus 229E, and animal coronaviruses, FIPV, and TGEV. Similarly, Sun *et al.* (2004) noted that the N protein of SARS-CoV reacts strongly not only with convalescent-phase SARS patient sera, but also with polyclonal antisera of known antigenic group I, including TGEV, FIPV, and canine CoV, yet does not cross-react with polyclonal antisera from antigenic group II (porcine HEV, and bovine CoV) or group III (turkey CoV and avian infectious virus) animal CoVs. Together, this data suggests that the N protein of SARS-CoV shares common antigenic epitopes with that of antigenic group I coronaviruses.

These findings raise potential concerns for the use of recombinant SARS-CoV N protein, whole virus antigen extracts, or virus-infected cells as reagents for diagnosis of SARS-CoV infection in humans and other animal species (Ksiazek *et al.,* 2003; Shi *et al.,* 2003; Guan *et al.,* 2004). Antigenic group I coronaviruses are known to infect a variety of human and animal species, thus, the use of N protein or whole virus as diagnostic antigens could produce a false-positive diagnosis of SARS, as evident in our study. Furthermore, the use of recombinant N protein or whole virus could complicate the search for a definitive natural reservoir of SARS-CoV as it is believed that SARS-CoV originated from wild animal species (Guan *et al.,* 2003; Martina *et al.,* 2003), many of which may have already been infected by known group I CoVs.

4.6 Cross-reactive region of SARS-CoV N protein

By aligning the N protein sequences of SARS-CoV with those of the other two human CoVs, a short region, corresponding to amino acids 111 to 118 (FYYLGTGP) of SARS-CoV, appears to be homologous amongst SARS-N, OC43-N, and 229E-N

(Appendix II). As this is the largest region of similarity between all three human CoVs and near the N-terminus, it was reasonable to hypothesize that this domain may be responsible for cross-reaction observed between human coronaviruses. Therefore, in an attempt to overcome the problematic cross-reaction, N-terminal truncated CoV N proteins were expressed in insect cells using the Baculovirus Expression System (Figure 21B).

Initial analysis of the C-terminal construct of SARS-CoV N by Western blot demonstrated that removal of the N-terminus appears to abolish cross-reaction between antibodies against human CoVs. This was evident by the elimination of false-positive responses to SARS-N from non-SARS sera (Figure 22, patient 1). After screening a larger population of sera by ELISA, false-positive results appeared, however, the rate of detection was lower for the truncated CoV N construct than that of the full-length SARS-CoV N protein (Table 6). The OC43 and 229E C-terminal constructs did not react with a corresponding 229E positive or OC43 positive patient and therefore not used further. The lack of reaction with positive sera may be due to the requirement for proper conformation for recognition. To clarify these results, competition assays were performed to determine if the C-terminal constructs of OC43-N or 229E-N could inhibit the binding of patient sera to the C-terminal SARS-N construct, thereby indicating the occurrence of cross-reaction. Several sera, including many seropositive non-SARS sera, experienced a decrease in binding to SARS-CoV (Figure 24B). Therefore, it appears that cross-reaction is still occurring amongst HCoVs, which may account for false-positive responses. Overall, removal of the N-terminus of SARS-N, containing a highly conserved motif between all HCoVs, reduced the apparent cross-reaction between HCoV nucleocapsid proteins. However, the continued incidence of cross-reaction suggests an alternative

region of homology is responsible for the remaining cross-reactivity. This may lie within the C-terminus as all three HCoVs share a short sequence of amino acids, KPRXKR.

In conclusion, the data demonstrated that the C-terminal construct of SARS-CoV N produces the greatest immune response during SARS-CoV infection, both in the early and late stage of the disease. Therefore, the recombinant SARS-CoV N protein based ELISA and Western blot assay are promising methods for early diagnosis of SARS-CoV infection.

4.7 Future Work

While this research shed valuable light on the human immune response to SARS-CoV infection and specificity of serological response to human coronaviruses, several avenues in this exciting area of research need to be addressed.

Firstly, our work identified a region responsible for a portion of cross-reaction between HCoV N proteins, however, it would be interesting to determine the exact region(s) responsible for this phenomenon. Generation of monoclonal antibodies, peptides, or truncated fragments spanning the entire SARS-CoV N protein could be used to further elucidate the regions of homology responsible for cross-reaction. Knowledge gained from this study could be used to identify an immunoreactive epitope within the N protein that has no antigenic cross-reactivity to known CoVs. Discovery of such a protein would be invaluable in SARS diagnosis and identification of the SARS-CoV animal reservoir.

Secondly, there are several proteins unique to SARS-CoV that have yet to be explored as potential antigenic and immunodominant proteins (ie. U70, U98, U84, U154, U274). Identification of such proteins will expand our knowledge on the human immune response to SARS-CoV infection and may aid in the development of diagnostic and therapeutic tools.

Thirdly, a recently identified human coronavirus, HCoV-NL63, has been detected worldwide in a significant number of respiratory tract illnesses of unknown etiology. As it has been placed in group I, alongside HCoV-229E, it would be interesting to try similar expression and serological specificity studies on HCoV-NL63 and HCoV-229E to

determine whether it will cross-react with antibodies from other human CoVs, as does HCoV-229E.

Lastly, it is important to thoroughly understand cellular and humoral immune responses to SARS-CoV, including the impact of SARS-CoV on immune function and any immunopathologic responses the virus may provoke. To date, the correlation between specific immune responses and subsequent clinical outcome remains unclear. In our study, the detection of SARS-specific antibodies was highly variable from one individual to another on the same day. However, recent investigations have suggested that the upsurge of IgG antibody to SARS-CoV correlates with the clinical worsening of pneumonia (Wang *et al.,* 2004; Peiris *et al.,* 2003). Further studies on the role of the immune system in SARS pathogenesis would help to identify correlates of protective immunity.

The ideas outlined in this section have the potential to further strengthen the results obtained from this study, and together they can provide data to expand our knowledge of the antigenic determinants of human coronaviruses and the human immune responses they evoke. This information will then serve as a foundation for the development of effective diagnostic tools and therapeutic interventions.

4.8 Conclusions

The following conclusions can be drawn from this study:

(1) SARS-CoV N protein is the most abundant protein found in SARS-CoV virions.

(2) SARS-CoV N protein is the immunodominant structural protein of SARS-CoV in humans.

(3) Anti-nucleocapsid IgG is the predominant antibody produced in response to SARS-CoV infection in humans.

(4) Immunodominant region of SARS-CoV N protein lies within the C-terminus.

(5) Cross-reaction occurs amongst antibodies against human coronavirus N proteins, primarily between SARS-CoV and HCoV-229E.

(6) Cross-reaction between anti-HCoV antibodies is, in part, dependent on amino acid homology found in the N-termini of the CoV N proteins.

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APPENDIX I

Primers and cloning strategies used for cloning CoV full-length (A) and truncated (B) proteins into pBlueBac4.5.
F = forward, R = reverse, ____ = coding sequence
1st nucleotide of PCR and RT-PCR primers are a clamp.
ATG = start codon, all stop codons have been removed

A) Primers for SARS, 229E, and OC43 CoV for pBlueBac4.5 constructs

CoV	Insert	Amino Acids	Primer Sequence (5' to 3')	Nucleotide Position	Method
SARS	N	1-421	GATGTCTGATAATGGACCCCAATC	1F	RT-PCR
			GTGCCTGAGTTGAATCAGCAGAAG	1266R	
	M	1-220	GATGGCAGACAACGGTACTATTAC	1F	RT-PCR
			GCTGTACTAGCAAAGCAATATTG	660R	
	E	1-75	GATGTACTCATTCGTTTCGGAAGAA	1F	RT-PCR
			GGACCAGAAGATCAGGAACTCCTTC	225R	
229E	N	1-389	GATGGCTACAGTCAAATGG	1F	RT-PCR
			GGTTTACTTCATCAATTATGTC	1167R	
OC43	N	1-448	GATGTCTTTTACTCCTGGT	1F	RT-PCR
			GTATTTCTGAGGTGTCTTC	1344R	

APPENDIX I con't

B) Primers for truncated CoV nucleocapsid fragments for pBlueBac4.5 constructs

CoV	Insert	Amino Acids	Primer Sequence (5' to 3')	Nucleotide Position	Method
229E	N-1	1-101	GATGGCTACAGTCAAATGG	1F	PCR
			GGACGACACCTTCAACACG	303R	
	N-3	88-389	GATGCATAAAGATGCAAAATTTAG	264F	
			GGTTTACTTCATCAATTATGTC	1167R	
OC43	N-5	1-145	GATGTCTTTTACTCCTGGT	1F	PCR
			GGACTCCGTCAATATCGGTGCC	435R	
	N-7	133-448	GATGCATGCTAAAGACCAGTACGGC	399F	
			GTATTTCTGAGGTGTCTTC	1344R	
Tor2	N-9	1-131	GATGTCTGATAATGGACCCCAA	1F	PCR
			GGATGCCTTCTTTGTTAGCGCC	393R	
	N-11	119-422	GATGGAAGCTTCACTTCCCTAC	357F	
			GTGCCTGAGTTGAATCAGCAGA	1266R	

APPENDIX II

Amino acid sequence of SARS-CoV (422 aa, black), HCoV-OC43 (448 aa, red), and HCoV-229E (389 aa, green) nucleocapsid protein. The sequences were aligned using Clustal V Method. The regions of homology are highlighted in grey.

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SARS    --------MSDNGPQSNQRSAPRITFGGPTDSTDNNQNGGRNGARPKQRRPQGLPNN-----TASWFTALTQHGK-EELRF  70
OC43    MSFTPGKQSSSRASSGNRSGNGILKWADQSDQFRNVQTRGRR-AQPKQTATSQQPSGGNVVPYYSWFSGITQFQKGKEFEF  80
229E    --------------------MATVKWADASE-----PQRGRQ----------GRIP-------YSLYSPLLVDSE-QPWKV  38

SARS    PRGQGVPINTNSGPDDQIGYYRRATRR-VRGGDGKMKELSPRWYFYYLGTGPEASLPYGANKEGIVWVATEGALNTPKDHI  147
OC43    VEGQGVPIAPGVPATEAKGYWYRHNRRSFKTADGNQRQLLPRWYFYYLGTGPHAKDQYGTDIDGVYWVASNQADVNTPADI  161
229E    IPRNLVPINKK-DKNKLIGYWNVQKR--FRTRKGKRVDLSPKLHFYYLGTGPHKDAKFRERVEGVVWVAVDGAKTEPTGYG  116

SARS    GTRNPNNN--AATVLQLPQGTTLPKGFYAEGSRGGSQASSRSSSRSRGNSRNSTPGS-SRGNSPARMASGGGETALALLLL  226
OC43    VDRDPSSD--EAIPTRFPPGTVLPQGYYIEGS-GRSAPNSRSTSRTS--SRASSAGSRSRANSGNRTPTSGVTPDMADQIA  240
229E    VRRKNSEPEIPHFNQKLPNGVTVVEEPDSRAP-SRSQSRSQSRGRGESKPQSRNPSS-DRNHNSQDDIMKAVAAALKSLGF  197

SARS    DRLNQLESKVSGKGQQQ----------------QGQTVTKKSAAEASKKPRQKRTATK--QYNVTQAFGRRGPEQTQGN  288
OC43    SLVLAKLGKDATK-PQQ----------------VTKHTAKEVRQKILNKPRQKRSPNK--QCTVQQCFGKRGPNQ---N  299
229E    DKPQEKDKKSAKTGTPKPSRNQSPASSQTSAKSLARSQSSETKEQKHEMQKPRWKRQPNDDVTSNVTQCFGPRDLDH---N  276

SARS    FGDQDLIRQGTDYKHWPQIAQFAPSASAFFGMSRIGMEVTPSGT----------WLTYHGAIKLDDKDPQFKDNVILLNK  367
OC43    FGGGEMLKLGTSDPQFPILAELAPTAGAFFFGSRLELAKVQNLSGNPDEPQKDVYELRYNGAIRFDSTLSGFETIMKVLNE  375
229E    FGSAGVVANGVKAKGYPQFAELVPSTAAMLFGSHIVSKESGNTV----------VLTFTTRVTVPKDHPHLG----KFLE  354

SARS    HIDAYKT------FPPTEPKKDKKKKTDEAQPLPQRQKKQPTVTLLPAADMDDFSRQLQNSMSGASADSTQA-  422
OC43    NLNAYQQQDGMMNMSPKPQRQRGHKNGQGENDNISVAVPKSRVQQNKSRELTAEDISLLKKMDEPYTEDTSEI  448
229E    ELNAFTR----------EMQQHPLLNPSALEFNPSQTSP---ATAEPVRDEVSIETDIIDEVN----------  389
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