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A Novel Coronavirus Associated with Severe Acute Respiratory Syndrome

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ABSTRACT

BACKGROUND

A worldwide outbreak of severe acute respiratory syndrome (SARS) has been associated with exposures originating from a single ill health care worker from Guangdong Province, China. We conducted studies to identify the etiologic agent of this outbreak.

METHODS

We received clinical specimens from patients in seven countries and tested them, using virus-isolation techniques, electron-microscopical and histologic studies, and molecular and serologic assays, in an attempt to identify a wide range of potential pathogens.

RESULTS

None of the previously described respiratory pathogens were consistently identified. However, a novel coronavirus was isolated from patients who met the case definition of SARS. Cytopathological features were noted in Vero E6 cells inoculated with a throat-swab specimen. Electron-microscopical examination revealed ultrastructural features characteristic of coronaviruses. Immunohistochemical and immunofluorescence staining revealed reactivity with group I coronavirus polyclonal antibodies. Consensus coronavirus primers designed to amplify a fragment of the polymerase gene by reverse transcription–polymerase chain reaction (RT-PCR) were used to obtain a sequence that clearly identified the isolate as a unique coronavirus only distantly related to previously sequenced coronaviruses. With specific diagnostic RT-PCR primers we identified several identical nucleotide sequences in 12 patients from several locations, a finding consistent with a point-source outbreak. Indirect fluorescence antibody tests and enzyme-linked immunosorbent assays made with the new isolate have been used to demonstrate a virus-specific serologic response. This virus may never before have circulated in the U.S. population.

CONCLUSIONS

A novel coronavirus is associated with this outbreak, and the evidence indicates that this virus has an etiologic role in SARS. Because of the death of Dr. Carlo Urbani, we propose that our first isolate be named the Urbani strain of SARS-associated coronavirus.

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In late 2002, cases of life-threatening respiratory disease with no identifiable cause were reported from Guangdong Province, China; they were followed by reports from Vietnam, Canada, and Hong Kong of severe febrile respiratory illness that spread to household members and health care workers. The syndrome was designated "severe acute respiratory syndrome" (SARS) in March 2003,[1-5] and global efforts to understand the cause of this illness and prevent its spread were instituted in March 2003. Many cases can be linked through chains of transmission to a health care worker from Guangdong Province, China, who visited Hong Kong, where he was hospitalized with SARS and died. Clinical specimens from patients meeting the case definition of SARS were sent to the Centers for Disease Control and Prevention (CDC) by collaborators in Vietnam, Singapore, Thailand, Hong Kong, Canada, Taiwan, and the United States as part of the etiologic investigation. In this report, we describe the efforts of the CDC to detect a wide range of possible etiologic agents for this disease outbreak, and we describe the identification and initial characterization of a novel coronavirus associated with cases of SARS.

METHODS

GENERAL APPROACH

The nonspecific nature of the clinical presentation of patients with SARS and the urgency of finding a cause required that clinical specimens be tested rapidly for a broad range of viral, bacterial, chlamydial, and rickettsial agents (the CDC case definition of SARS is available as Supplementary Appendix 1 with the full text of this article at http://www.nejm.org). Laboratory testing focused foremost on known respiratory pathogens, especially those that might specifically target the lower respiratory tract through the progression of disease. A combination of traditional methods was applied, including virus isolation in suckling mice and cell culture, electron microscopy, histopathological examination, serologic analysis, and general and specialized bacterial culture techniques. The molecular techniques of polymerase chain reaction (PCR), reverse-transcription PCR (RT-PCR), and real-time PCR were used. Priority was given to testing for the following agents: yersinia, mycoplasma, chlamydia, legionella, *Coxiella burnetii*, spotted fever and typhus group rickettsiae, influenzaviruses A and B, Paramyxovirinae and Pneumovirinae subfamily viruses (specifically, human respiratory syncytial virus and human metapneumovirus), Mastadenoviridae, Herpetoviridae, Picornaviridae, Old and New World hantaviruses, and Old World arenaviruses.

BIOSAFETY

Given the serious nature of SARS and the suggestion of person-to-person transmission, it was decided to handle all clinical specimens in a biosafety level 3 environment. All division into aliquots, pipetting, and culture attempts were performed in laminar-flow safety cabinets in a biosafety level 3 laboratory. Serum specimens that were tested serologically outside the laboratory were exposed to ^{60}Co gamma irradiation at 2×10^6 rad while frozen on dry ice. Initial division into aliquots, handling, and culturing were undertaken in a biosafety level 3 laboratory area in which no culturing of known viruses is done. A similar environment was used when specimens from which nucleic acid was to be extracted were placed in a solution of chaotropic salts; after this step, the specimens were removed to other areas for completion of the extraction protocols.

ISOLATION OF VIRUS

To identify viruses associated with SARS, we inoculated a variety of clinical specimens (blood, serum, material from oropharyngeal swabs or washings, material from nasopharyngeal swabs, and tissues of major organs collected at autopsy) onto a number of continuous cell lines, including Vero E6, NCI-H292, MDCK, LLC-MK2, and B95-8 cells, and into suckling ICR mice by the intracranial and intraperitoneal routes. All cultures were observed daily for cytopathic effect. Maintenance medium was replenished at day 7, and cultures were terminated 14 days after inoculation. Any cultures exhibiting identifiable cytopathic effect were subjected to several procedures to identify the cause of the effect. Suckling mice were observed daily for 14 days, and we further tested any sick or dead mice by preparing a brain suspension that was filtered and subcultured. Mice that remained well after 14 days were euthanized, and their test results were recorded as negative. Tissue-culture samples showing cytopathic effect were prepared for electron-microscopical examination. Negative-stain electron-microscopical specimens were prepared by drying culture supernatant, mixed 1:1 with 2.5 percent paraformaldehyde, onto Formvarcarbon-coated grids and staining

with 2 percent methylamine tungstate. Thin-section electron-microscopical specimens were prepared by fixing a washed cell pellet with 2.5 percent glutaraldehyde and embedding it in epoxy resin. For RT-PCR assays, cell-culture supernatants were placed in lysis buffer. In addition, a master seed was prepared from the remaining culture supernatant and cells by freeze-thawing the culture flask, clarifying the thawed contents by centrifugation at 1000×*g*, and dispensing the supernatant into aliquots stored in gas phase over liquid nitrogen. The master seed was subcultured into 850-cm² roller bottles of Vero E6 cells for the preparation of formalin-fixed positive control cells for immunohistochemical analysis, mixed with normal E6 cells, and gamma-irradiated for preparation of spot slides for indirect fluorescence antibody tests or extracted with detergent and gamma-irradiated for use as an enzyme-linked immunosorbent assay (ELISA) antigen for antibody tests.

SEROLOGIC ANALYSIS

Spot slides were prepared by applying 15 μl of the suspension of gamma-irradiated mixed infected and noninfected cells onto 12-well Teflon-coated slides. Slides were allowed to air dry before being fixed in acetone. Slides were then stored at −70°C until used for indirect fluorescence antibody tests.[6] An ELISA antigen was prepared by detergent extraction of infected Vero E6 cells and subsequent gamma irradiation.[7] The optimal dilution (1:1000) for the use of this antigen was determined by checkerboard titration against serum from a patient with SARS in the convalescent phase; a control antigen, similarly prepared from uninfected Vero E6 cells, was used to control for specific reactivity of tested serum. The conjugates used were goat antihuman IgG, IgA, and IgM conjugated to fluorescein isothiocyanate and horseradish peroxidase (Kirkegaard and Perry), for the indirect fluorescence antibody test and ELISA, respectively. Specificity and cross-reactivity of a variety of serum samples to the newly identified virus were evaluated by using the tests described above. For this evaluation, we used serum from patients with SARS in Singapore, Bangkok, and Hong Kong and serum from healthy blood donors from the CDC serum bank and from persons infected with known human coronaviruses (human coronaviruses OC43 and 229E) (samples provided by E. Walsh and A. Falsey, University of Rochester School of Medicine and Dentistry, Rochester, N.Y.).

PATHOLOGICAL AND IMMUNOHISTOCHEMICAL STUDIES

Formalin-fixed, paraffin-embedded Vero E6 cells infected with the novel coronavirus and tissues obtained from patients with SARS were stained with hematoxylin and eosin and various immunohistochemical stains. Immunohistochemical assays were based on a method described previously for hantavirus.[8] In brief, 4-μm sections were deparaffinized, rehydrated, and digested in Proteinase K for 15 minutes. Slides were then incubated for 60 minutes at room temperature with monoclonal antibodies, polyclonal antiserum, or ascitic fluids derived from animal species with reactivities to various known coronaviruses, and with a convalescent-phase serum specimen from a patient with SARS.

Optimal dilutions of the primary antibodies were determined by titration experiments with cells infected with the new coronavirus and with noninfected cells or, when available, with concentrations recommended by the manufacturers. After sequential application of the appropriate biotinylated link antibody, avidin–alkaline phosphatase complex, and naphthol–fast red substrate, sections were counterstained in Mayer's hematoxylin and mounted with aqueous mounting medium. We used the following antibody and tissue controls: serum specimens from noninfected animals, various coronavirus-infected cell cultures and animal tissues, noninfected cell cultures, and normal human and animal tissues. Tissues from patients were also tested by immunohistochemical assays for various other viral and bacterial pulmonary pathogens. In addition, a bronchoalveolar-lavage specimen was available from one patient with SARS for thin-section electron-microscopical evaluation.

MOLECULAR ANALYSES

RNA extracts were prepared from 100 μl of each specimen (or culture supernatant) with the automated NucliSens extraction system (bioMérieux). Oligonucleotide primers used for amplification and sequencing of the SARS-related coronavirus were designed from alignments of open reading frame 1b of the coronavirus polymerase gene sequences obtained from GenBank, including human coronaviruses 229E and OC43 (accession numbers X69721 and AF124989, respectively), canine coronavirus (AF124986), feline infectious peritonitis virus (AF124987), porcine transmissible gastroenteritis virus (Z34093), porcine epidemic diarrhea virus

(NC_003436), bovine coronavirus (NC_003045), porcine hemagglutinating encephalomyelitis virus (AF124988), sialodacryoadenitis virus (AF124990), mouse hepatitis virus (NC_001846), turkey coronavirus (AF124991), and avian infectious bronchitis virus (NC_001451). Primer pair IN-2 (+) 5′GGGTTGGGACTATCCTAAGTGTGA3′ and IN-4 (–) 5′TAACACACAACICCATCATCA3′ was previously designed to conserved regions of open reading frame 1b to achieve broad reactivity with the genus coronavirus. These primers were used to amplify DNA from SARS isolates, and the amplicon sequences obtained were used to design SARS-specific primers Cor-p-F2 (+) 5′CTAACATGCTTAGGATAATGG3′, Cor-p-F3 (+) 5′GCCTCTCTTGTTCTTGCTCGC3′, and Cor-p-R1 (–) 5′CAGGTAAGCGTAAAACTCATC3′, which were used in turn to test patient specimens. Primers used for specific amplification of human metapneumovirus have been described previously.[9] Well-characterized primer sets for other respiratory virus pathogens (unpublished data), including human respiratory syncytial virus, parainfluenzaviruses 1, 2, and 3, influenzaviruses A and B, adenovirus, and picornavirus (rhinovirus and enterovirus), were also used to test clinical specimens in this study (primers available on request). All specimens were tested for human glyceraldehyde-3-phosphate dehydrogenase to confirm RNA integrity and control for RT-PCR inhibition.

One primer for each set was 5′-end-labeled with fluorescent dye 6-carboxyfluorescein (6-FAM) to facilitate GeneScan analysis. One-step amplification reactions were performed with the Access RT-PCR System (Promega) as previously described.[9] Positive and negative RT-PCR controls, containing standardized viral RNA extracts, and nuclease-free water were included in each run. Amplified 6-FAM–labeled products were analyzed by capillary electrophoresis on an ABI 3100 Prism Genetic Analyzer with GeneScan software (version 3.1.2). Specimens were considered positive for SARS-associated coronavirus if the amplification products were within 1 nucleotide of the expected product size (368 nucleotides for Cor-p-F2 or Cor-p-R1 and 348 nucleotides for Cor-p-F3 or Cor-p-R1) for both specific primer sets, as confirmed by a second PCR reaction from another aliquot of RNA extract in a separate laboratory. Where DNA yield was sufficient, the amplified products were also sequenced. The microarray used in this study was an expanded version of an array previously described.[10,11]

For sequencing, amplicons were purified with ExoSAP-IT (USB). Both strands of unlabeled products (or one strand of the 6-FAM–labeled products) were sequenced on an ABI PRISM 3100 Genetic Analyzer with use of a fluorescent dye-terminator kit (ABI). The nucleotide sequences were edited with Sequencher for Power Macintosh (version 3.1.1, Gene Codes). The partial nucleotide sequences of the polymerase gene were aligned with published coronavirus sequences, using CLUSTAL W for Unix (version 1.7).[12] Phylogenetic trees were computed by maximum parsimony, distance, and maximum likelihood–based criteria analysis with PAUP (version 4.0.d10).[13]

RESULTS

VIRUS ISOLATION

Two cell lines, Vero E6 cells and NCI-H292 cells, inoculated with oropharyngeal specimens from Patient 16 (a 46-year-old male physician with an epidemiologic link to a hospital with multiple patients with SARS) initially showed cytopathic effect (Table 1). Blood, nasopharyngeal, and throat-swab specimens were collected on March 12, day 1 after onset. At that time, the patient's physical examination was normal except for fever and shortness of breath. During the course of the disease, his status worsened, and he died. A rhinovirus was isolated from the inoculated NCI-H292 cells. Further study suggested that this virus was not associated with patients with SARS, so it will not be discussed here.

Cytopathic effect in the Vero E6 cells was first noted on the fifth post-inoculation day. The cytopathic effect was focal, with cell rounding and a refractive appearance in the affected cells (Fig. 1) that was soon followed by cell detachment. The cytopathic effect quickly spread to involve the entire cell monolayer within 24 to 48 hours. Subculture of material after preparation of a master seed resulted in the rapid appearance of cytopathic effect, as noted above, and in complete destruction of the monolayer in the inoculated flasks within 48 hours. Similar cytopathic effect has since been noted in four additional cultures: three cultures of respiratory specimens (two oropharyngeal washes and one sputum specimen) and one culture of a suspension of kidney tissue obtained at autopsy. In these specimens, the initial cytopathic effect was observed between day 2 and day 4 and, as noted above, the cytopathic effect rapidly progressed to involve the entire cell monolayer.

Examination of cytopathic-effect–positive Vero

Table 1. Specimens from Patients with SARS That Were Positive for SARS-Associated Coronavirus by One or More Methods.*

Patient No.	Exposure and Setting	Age/Sex	Findings on Chest Radiograph	Hospitalization	Serologic Results	Specimen	Isolation	RT-PCR
1	Singapore, hospital	53 yr/F	Pneumonia	Yes	+	Nasal, oropharyngeal swabs	–	Not done
2†	Hong Kong, hotel	36 yr/F	Pneumonia	Yes	+	Nasal, swab	–	Not done
3	Hong Kong, hotel	22 yr/M	Pneumonia	Yes	+	Swab	–	–
4†	Hong Kong, hotel	39 yr/M	Pneumonia	Yes	+	Nasal, pharyngeal swab	–	–
5	Hong Kong, hotel	49 yr/M	Pneumonia	Yes	Not done	Sputum	+	+
6‡	Hong Kong, hotel	46 yr/M	Pneumonia	Yes	+	Kidney, lung, bronchoalveolar lavage	+§	+
7	Vietnam, hospital	Adult/unknown	Pneumonia	Yes	–	Oropharyngeal wash	+	+
8	Vietnam, hospital	Adult/unknown	Pneumonia	Yes	–	Oropharyngeal wash	–	+
9	Vietnam, hospital	Adult/unknown	Pneumonia	Yes	–	Oropharyngeal wash	–	+
10	Vietnam, hospital	Adult/unknown	Pneumonia	Yes	–	Oropharyngeal wash	–	+
11	Vietnam, hospital	Adult/unknown	Pneumonia	Yes	–	Oropharyngeal wash	–	+
12	Vietnam, hospital	Adult/unknown	Pneumonia	Yes	–	Oropharyngeal wash	–	+
13	Vietnam, hospital	Adult/unknown	Pneumonia	Yes	–	Oropharyngeal wash	+	+
14	Vietnam, hospital	Adult/unknown	Pneumonia	Yes	–	Oropharyngeal wash	–	+
15	Vietnam, hospital	Adult/unknown	Pneumonia	Yes	–	Oropharyngeal wash	–	+
16	Vietnam, hospital	46 yr/M	Pneumonia	Yes	+	Nasal, oropharyngeal swab	+¶	+
17	Canada, family	43 yr/M	Pneumonia	Yes	Not done	Lung, bone marrow	–	+
18	Taiwan, family	51 yr/F	Pneumonia	Yes	–	Sputum	–	+
19	Hong Kong, hotel	Adult/F	Pneumonia	Yes	+	Oropharyngeal wash	–	+

* Plus signs denote positive results, and minus signs negative results. The serologic and RT-PCR assays were not necessarily performed on samples obtained at the same time.
† This was a late specimen, antibody positive at first sample.
‡ Travel included China, Hong Kong (hotel), and Hanoi (the patient was the index patient in the French Hospital).
§ Isolation was from the kidney only.
¶ Isolation was from the oropharyngeal specimen only.

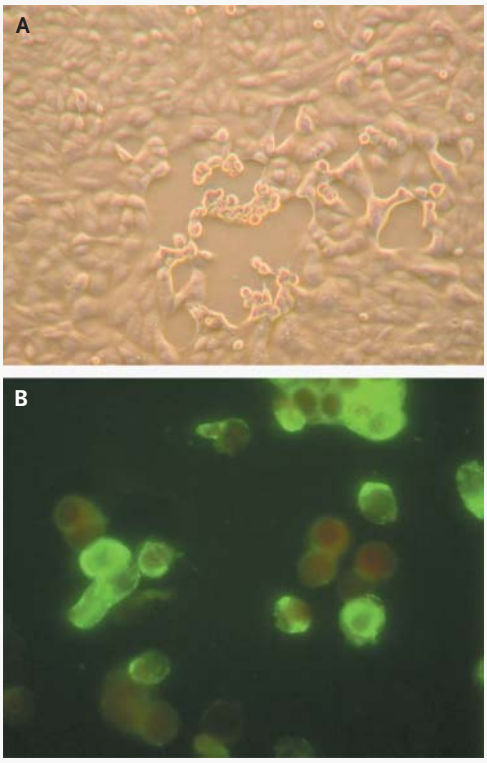

Figure 1. Vero E6 cells Inoculated with Oropharyngeal Specimens from Patients with SARS.

The typical early cytopathic effect seen with coronavirus isolates from patients with SARS is shown in Panel A (×40). Infected Vero cells are shown reacting with the serum of a convalescent patient in an indirect fluorescence antibody assay in Panel B (×400).

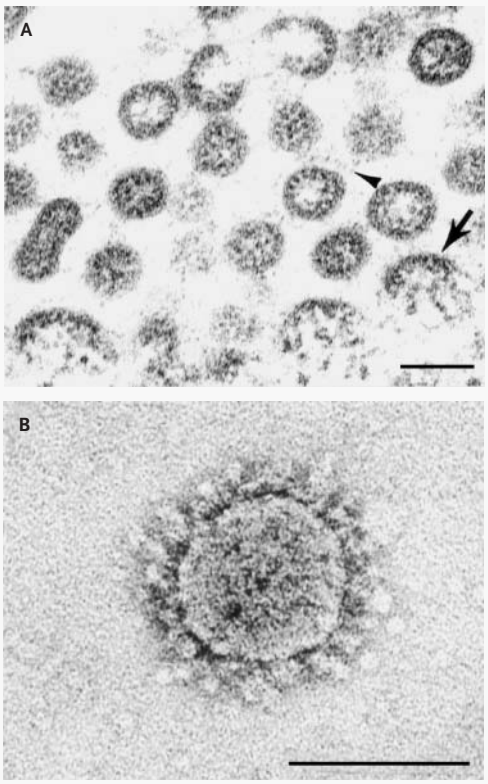

Figure 2. Ultrastructural Characteristics of SARS-Associated Coronavirus Grown in Vero E6 Cells.

Panel A shows a thin-section electron-microscopical view of viral nucleocapsids aligned along the membrane of the rough endoplasmic reticulum (arrow) as particles bud into the cisternae. Enveloped virions have surface projections (arrowhead) and an electron-lucent center. Directly under the viral envelope lies a characteristic ring formed by the helical nucleocapsid, often seen in cross section. Negative-stain electron microscopy (Panel B) shows a stain-penetrated coronavirus particle with an internal helical nucleocapsid-like structure and club-shaped surface projections surrounding the periphery of the particle, a finding typical of coronaviruses (methylamine tungstate stain). The bars represent 100 nm.

E6 cells by thin-section electron microscopy revealed characteristic coronavirus particles within the cisternae of the rough endoplasmic reticulum and in vesicles (Fig. 2A).[14,15] Extracellular particles were found in large clusters and adhering to the surface of the plasma membrane. Negative-stain electron microscopy identified coronavirus particles, 80 to 140 nm in diameter, with 20-to-40-nm complex surface projections surrounding the periphery (Fig. 2B). Hemagglutinin esterase-type glycoprotein projections were not seen.

MOLECULAR ANALYSIS

A 405-nucleotide segment of the coronavirus polymerase gene open reading frame 1b was amplified from the isolation material by RT-PCR with the broadly reactive primer set IN-2–IN-4. In contrast, this primer set produced no specific band against uninfected cells.

When compared with other human and animal coronaviruses, the nucleotide and deduced amino acid sequence from this region had similarity scores ranging from 0.56 to 0.63 and from 0.57 to 0.74, respectively. The highest sequence similarity was obtained with group II coronaviruses. The maximum-parsimony tree obtained from the nucleotide-sequence alignment is shown in Figure 3. Boot-

strap analyses of the internal nodes at the internal branches of the tree provided strong evidence that the SARS-associated coronavirus is genetically distinct from other known coronaviruses. The microarray analyses from infected and uninfected cell cultures gave a positive signal for a group of eight oligonucleotides derived from two virus families: Coronaviridae and Astroviridae. All of the astroviruses and two of the coronavirus oligonucleotides share a consensus sequence motif that maps to the extreme 3′ end of astroviruses and two members of the coronavirus family: avian infectious bronchitis and turkey coronavirus.[16] Results were consistent with the identity of the isolate as a coronavirus.

IMMUNOHISTOCHEMICAL AND HISTOPATHOLOGICAL ANALYSIS AND ELECTRON-MICROSCOPICAL ANALYSIS OF BRONCHOALVEOLAR-LAVAGE FLUID

Lung tissues were obtained at autopsy from three patients and by open-lung biopsy in one patient, 14 to 19 days after the onset of SARS. Confirmatory laboratory evidence of infection with coronavirus was available for two patients (Patients 6 and 17) and included PCR amplification of coronavirus nucleic acids from tissues, viral isolation from bronchoalveolar-lavage fluid, or detection of serum antibodies reactive with coronavirus (Table 1). For two patients, no samples were available for molecular, cell-culture, or serologic analysis; however, the condition of both patients met the CDC definition of probable SARS, and both had strong epidemiologic links with laboratory-confirmed cases of SARS. Histopathological evaluation of lung tissues from the four patients showed diffuse alveolar damage at various levels of progression and severity. Changes included hyaline-membrane formation, interstitial mononuclear inflammatory infiltrates, and desquamation of pneumocytes in alveolar spaces (Fig. 4A). Other findings identified in some patients included focal intraalveolar hemorrhage, necrotic inflammatory debris in small airways, and organizing pneumonia. Multinucleated syncytial cells were identified in the intraalveolar spaces of two patients who died 14 and 17 days after onset of illness. These cells contained abundant vacuolated cytoplasm with cleaved and convoluted nuclei. No obvious intranuclear or intracytoplasmic viral inclusions were identified (Fig. 4B), and electron-microscopical examination of a limited number of these syncytial cells revealed no coronavirus particles. No definitive immunostaining was identified in tissues from SARS patients with the use of a battery of immunohistochemical stains reactive with coronaviruses from antigenic groups I, II, and III. In addition, no staining of patient tissues was identified with the use of immunohistochemical stains for influenzaviruses A and B, adenoviruses, Hendra and Nipah viruses, human metapneumovirus, respiratory syncytial virus, measles virus, *Mycoplasma pneumoniae*, and *Chlamydia pneumoniae*.

Evaluation of Vero E6 cells infected with coronavirus isolated from a patient with SARS revealed

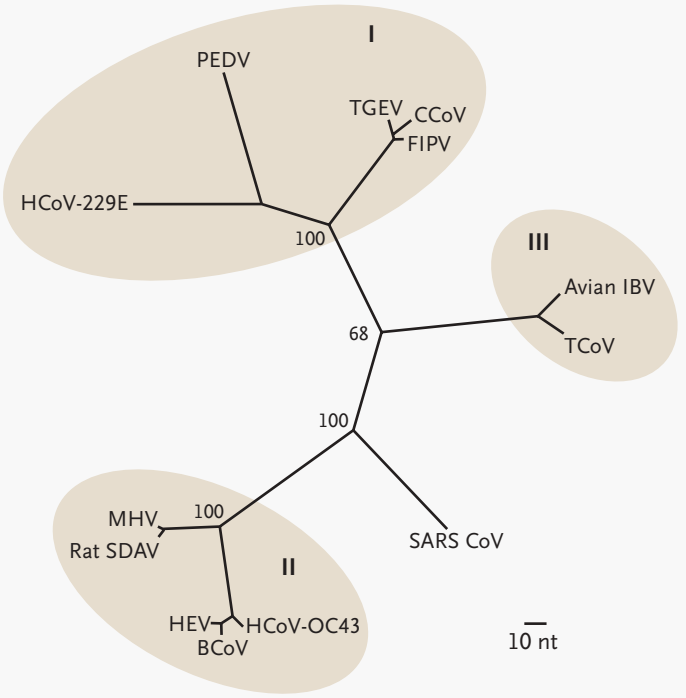

Figure 3. Estimated Maximum-Parsimony Tree Based on the Sequence Alignment of 405 Nucleotides of the Coronavirus Polymerase Gene Open Reading Frame 1b (Nucleotide Numbers 15173 to 15578 Based on Bovine Coronavirus Complete Genome Accession Number NC_003045) Comparing SARS Coronavirus with Other Human and Animal Coronaviruses.

The three major coronavirus antigenic groups (I, II, and III), 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 encephalomyelitis virus (HEV), rat sialodacryoadenitis virus (SDAV), mouse hepatitis virus (MHV), turkey coronavirus (TCoV), and avian infectious bronchitis virus (IBV), are shown shaded. Bootstrap values (from 100 replicates) obtained from a 50 percent majority rule consensus tree are plotted at the main internal branches of the phylogram. Branch lengths are proportionate to nucleotide differences.

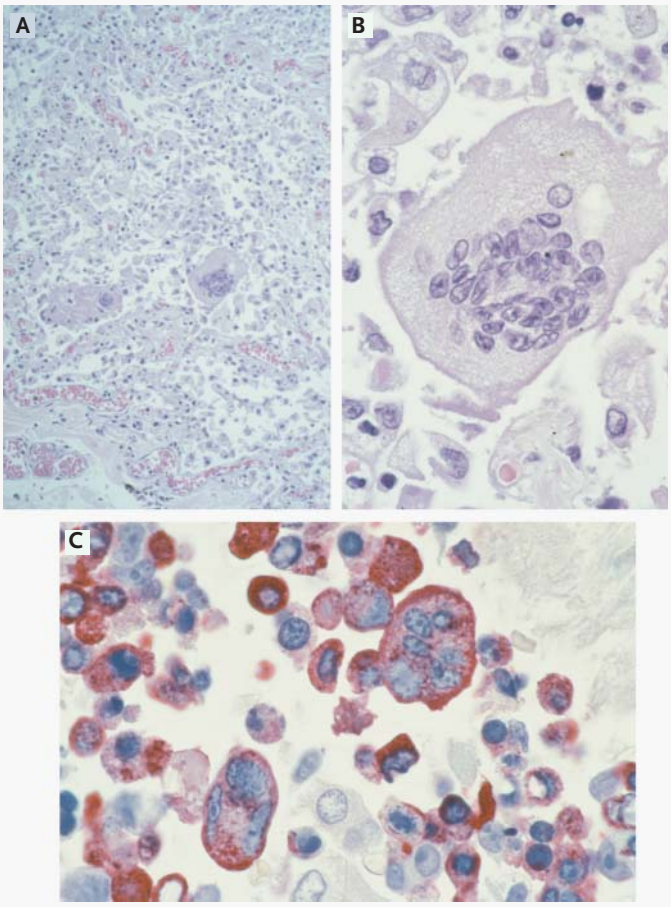

Figure 4. Histopathological Evaluation of Lung Tissue from a Patient with SARS and Immunohistochemical Staining of Vero E6 Culture Cells Infected with SARS-Associated Coronavirus.

Panel A shows diffuse alveolar damage, abundant foamy macrophages, and multinucleated syncytial cells. Panel B shows a higher magnification of a pulmonary syncytial cell with no conspicuous viral inclusions. Panel C shows immunohistochemical staining of SARS-associated coronavirus–infected culture cells. Membranous and cytoplasmic immunostaining of individual and syncytial Vero E6 cells is demonstrated with a cat anti–feline infectious peritonitis virus 1 ascitic fluid. (Panels A and B, hematoxylin and eosin, ×50 and ×250, respectively; Panel C, immunoalkaline phosphatase with naphthol–fast red substrate and hematoxylin counterstain, ×250).

viral cytopathic effect that included occasional multinucleated syncytial cells but no obvious viral inclusions (Fig. 4C). Immunohistochemical assays with various antibodies reactive with coronaviruses from antigenic group I, including human coronavirus 229E, feline infectious peritonitis virus 1, and porcine transmissible gastroenteritis virus, and with an immune serum specimen from a patient with SARS demonstrated strong cytoplasmic and membranous staining of infected cells (Fig. 4C and Table 2); however, cross-reactivity with the same immune human serum sample and feline infectious peritonitis virus 1 antigen was not observed. No staining was identified with any of several monoclonal or polyclonal antibodies reactive with coronaviruses in antigenic group II (human coronavirus OC43, bovine coronavirus, and mouse hepatitis virus) or group III (turkey coronavirus and avian infectious bronchitis virus). Electron-microscopical examination of bronchoalveolar-lavage fluid from one patient revealed many coronavirus-infected cells (Fig. 5).

SEROLOGIC ANALYSIS

Spot slides with infected cells reacted with serum from patients with probable SARS in the convalescent phase. Screening of a panel of serum from patients with suspected SARS from Hong Kong, Bangkok, Singapore, and the United States showed a high level of specific reaction with infected cells and conversion from negative to positive reactivity or diagnostic rises in the indirect fluorescence antibody test by a factor of four. Similarly, tests of these same serum samples with the ELISA antigen showed high specific signal in the convalescent-phase samples and conversion from negative to positive antibody reactivity or diagnostic increases in titer (Table 3). Information from the limited number of samples tested thus far suggests that antibody is first detectable in these two tests between one and two weeks after the onset of symptoms in the patient. Indirect fluorescence antibody testing and ELISA of a panel of 384 randomly selected serum samples (from U.S. blood donors) were negative for antibodies to the new coronavirus, with the exception of 1 specimen that had minimal reactivity on ELISA. A panel of paired human serum samples with diagnostic increases (by a factor of four or more) in antibody (with very high titers to the homologous viral antigen in the convalescent-phase serum) to the two known human coronaviruses, OC43 (13 pairs) and 229E (14 pairs), showed no reactivity in either acute- or convalescent-phase serum with the newly isolated coronavirus by either the indirect fluorescence antibody test or the ELISA.

PATIENTS

Nineteen patients with SARS have been identified as infected with the new coronavirus by virus isolation, RT-PCR, or serologic tests; all have direct or indirect links to the SARS outbreak in Hong Kong or Guang-

dong Province, China (Table 1). We were able to amplify by RT-PCR and obtain the virus sequence from clinical specimens or virus isolates from 12 of these patients. All 12 sequences were identical to those of the first isolate as noted above. For four convalescent patients, infection was detected serologically alone; for nine patients it was detected by RT-PCR alone; for three by virus isolation and RT-PCR; for two by virus isolation, RT-PCR, and serologic analysis; and for one by RT-PCR and serologic analysis. We found none of the coronavirus-infected patients to be infected with human metapneumovirus. In only one patient was both SARS coronavirus and another respiratory virus detected; Patient 16 had both SARS coronavirus and a rhinovirus. A variety of respiratory pathogens were also identified by RT-PCR in other patients whose samples were submitted for SARS testing, including 5 with human metapneumoviruses (sequencing showed that each was distinct) and 12 with rhinoviruses (sequencing showed that each was distinct). None of the patients who were positive for human metapneumovirus had pneumonia.

Table 2. Immunohistochemical Reactivities of Various Polyclonal Group I Anti-Coronavirus Reference Antiserum Samples with a Coronavirus Isolated from a Patient with SARS and with Selected Antigenic Group I Coronaviruses.*

Antiserum	Immunohistochemical Reactivity of Antiserum with Coronavirus-Infected Culture Cells (Cell Line)		
	SARS CoV (Vero E6)	HCoV-229E (3T3-hAPN)	FIPV-1 (BHK-fAPN)
Convalescent-phase SARS serum (Patient 3)	+	+	–
Guinea pig anti–HCoV-229E	+	+	–
Rabbit anti–HCoV-229E	+	+	+
Feline anti-FIPV-1	+	+	+
Porcine anti-TGEV	+	–	+

* No reactivity of the novel coronavirus isolate (200300592) was identified with polyclonal or monoclonal antibodies reactive with the following viral antigens: FIPV-2, HCoV-OC43, MHV, BCoV, TCoV, or IBV. CoV denotes coronavirus, HCoV human coronavirus, FIPV feline infectious peritonitis virus, and TGEV transmissible gastroenteritis virus.

DISCUSSION

The isolation of a novel coronavirus from the respiratory secretions of a patient with SARS and the subsequent demonstration of this virus or a serologic response to this virus in others with SARS demonstrate an etiologic association between this virus and SARS. The discovery of this new virus occurred through a broad-based and multidisciplinary effort by clinical, epidemiologic, and laboratory investigators and speaks to the power of a global collaborative effort to address the ever-present threat of emerging infectious diseases.[17]

The three known groups of coronavirus are associated with a variety of diseases of humans and domestic animals, including gastroenteritis and upper and lower respiratory tract disease. Although the known human coronaviruses are associated with a mild disease (the common cold), the ability of coronavirus of animals to cause severe disease raises the possibility that coronavirus could also cause more severe disease in humans. Other than rare instances in children or immunocompromised patients, it appears that the SARS-related coronavirus may be the first example of a coronavirus that causes severe disease in humans. The novel human coronavirus identified in this study shares antigenic features with various group I coronaviruses, but genetic comparisons suggest it is distinct from group I coronaviruses and from coronaviruses in groups II and III. The factor or factors responsible for this apparent dichotomy remain to be elucidated; however, correlation between antigenic and genetic characteristics of these viruses is occasionally unclear, and the placement of some other human coronaviruses within specific antigenic groups has not always been well defined.[18-20]

The identification of this novel coronavirus relied on classic tissue-culture isolation to amplify the pathogen and then on electron-microscopical studies to identify the type of virus, a member of the family Coronaviridae, and molecular studies to confirm the identity of the virus, characterize its unique nature, and help link it to the disease. The discovery of this new virus underscores the importance of versatile techniques such as virus isolation and electron microscopy in identifying etiologic pathogens. As with previous outbreak investigations, electron microscopy proved to be a rapid technique that did not require specific reagents for or prior knowledge of a particular agent but that could nevertheless categorize a pathogen on the basis of its appearance and morphogenesis.[21-24]

In this report, we describe infection in 19 SARS

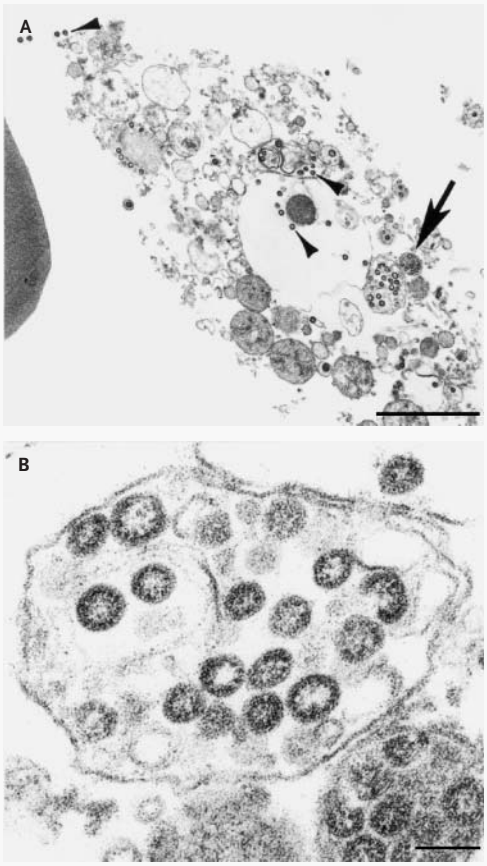

Figure 5. Ultrastructural Characteristics of a Coronavirus-Infected Cell in Bronchoalveolar-Lavage Fluid from a Patient with SARS, with Numerous Intracellular and Extracellular Particles.

The virions are indicated by the arrowheads in Panel A. Panel B shows the area indicated by the arrow in Panel A at higher magnification. The bar in Panel A represents 1 μm, and that in Panel B, 100 nm.

patients with well-defined direct or indirect epidemiologic links either to the outbreak in Hong Kong or to Guangdong Province, China, the origin of the index patient in Hong Kong. As expected with a point-source outbreak, the sequences from a limited region of the polymerase gene are identical. Coronaviruses with identical sequences have also been detected in patients with SARS in Canada.[5] The virus was found in multiple specimens, including lung and kidney tissue extracts by virus isolation or RT-PCR, bronchoalveolar-lavage specimens by electron microscopy and PCR, and sputum or upper respiratory tract swab, aspirate, or wash specimens by RT-PCR or virus isolation. Although we tested specimens from the coronavirus-positive patients for a variety of other respiratory pathogens, including human metapneumovirus, by RT-PCR, none were detected in these coronavirus-positive patients except for a rhinovirus in Patient 16. The relation between this novel coronavirus and disease is further evidenced by detection of virus in lung tissue and a bronchoalveolar-lavage specimen, thus placing the virus at the site of diseased tissue. We were not, however, able to demonstrate coronavirus antigens in patient tissues by histologic and immunohistochemical methods or to demonstrate a direct involvement in the pathologic process. Neither were we able to demonstrate SARS-associated coronavirus infection in all suspected patients with SARS.

Possible reasons for the inability to demonstrate infection in some patients with suspected SARS include the lack of sufficient sensitivity of the assays to detect the pathogen and the immune response and the timing and type of specimens tested. For example, we have not yet received convalescent-phase serum specimens from many patients with suspected SARS and have not serologically ruled out infection in many such patients. In addition, we are just beginning to study the type and timing of clinical specimens most likely to support a diagnosis of infection with this new virus. We have made rapid progress in developing our diagnostic assays and are continuing to improve them for the detection of this virus or an immune response to it. In addition, the case definition of SARS is very broad and most likely includes other infectious diseases. We are also continuing to test for other infectious agents that might be associated with SARS, including those that might contribute to the severity of disease or increase the efficiency of viral transmission. Further clinical analysis of patients with SARS in whom there is laboratory confirmation of infection with the new coronavirus might help refine the case definition further.

The apparent lack of antibody in all serum specimens except those from patients with SARS suggests that this virus has not previously circulated in humans. Certainly, it has not circulated widely in humans, which is further evidence of an association between infection with this novel coronavirus and SARS. Presumably, this virus originated in animals and mutated or recombined in a fashion that permitted it to infect, cause disease, and pass from person to person. The available sequence data for this novel coronavirus suggest that it is distinct from

Table 3. Results of Serologic Testing with Both Indirect Fluorescence Antibody (IFA) Test and Indirect Enzyme-Linked Immunosorbent Assay (ELISA) in Patients with SARS Tested against a Newly Isolated Coronavirus (200300592).

Location of Patient	Patient and Serum No.*	Number of Days after Onset Sample Obtained	ELISA Titer†	IFA Titer†
Hong Kong	1.1	4	<100	<25
Hong Kong	1.2	13	≥6400	1600
Hong Kong	2.1	11	400	100
Hong Kong	2.2	16	1600	200
Hong Kong	3.1	7	<100	<25
Hong Kong	3.2	17	≥6400	800
Hong Kong	4.1	8	<100	<25
Hong Kong	4.2	13	1600	50
Hong Kong	5.1	10	100	<25
Hong Kong	5.2	17	≥6400	1600
Hong Kong	6.1	12	1600	200
Hong Kong	6.2	20	≥6400	6400
Hong Kong	7.1	17	400	50
Hong Kong	7.2	24	≥6400	3200
Hong Kong	8.1	3	<100	<25
Hong Kong	8.2	15	≥6400	200
Hong Kong (Hanoi)	9.1	5	<100	<25
Hong Kong	9.2	11	≥6400	1600
Bangkok	1.1	2	<100	<25
Bangkok	1.2	4	<100	<25
Bangkok	1.3	7	<100	<25
Bangkok	1.4	15	1600	200
United States	1.1	2	<100	<25
United States	1.2	6	400	50
United States	1.3	13	≥6400	800
Singapore	1.1	2	100	<25
Singapore	1.2	11	≥6400	800
Singapore	2.1	6	100	<25
Singapore	2.2	25	≥6400	400
Singapore	3.1	6	100	<25
Singapore	3.2	14	≥6400	400
Singapore	4.1	5	100	<25
Singapore	4.2	16	1600	400

* Each number shown is the patient number and the number of the sample from that patient.
† The value is the reciprocal of the dilution.

those previously reported in animals and humans and that the parent virus has not yet been discovered. The isolation and growth of a human-derived coronavirus in Vero E6 cells were unexpected. The only human or animal coronavirus that has been shown to grow in Vero cells is porcine epidemic diarrhea virus (isolated in China from pigs), and it requires the addition of trypsin to culture medium for growth in Vero E6 cells.[25] However, like the sequences of the other known coronaviruses, the sequences for porcine epidemic diarrhea virus are distinct from those of SARS-associated coronavirus, indicating that porcine epidemic diarrhea virus is not the parent virus to this new coronavirus. Because of the death of Dr. Carlo Urbani during the investigation, we propose that our first isolate be named the Urbani strain of SARS-associated coronavirus.

Pathological studies in patients who died with SARS show diffuse alveolar damage as the most notable feature, a finding consistent with the severe respiratory illness seen in some patients with SARS. The primary histopathological lesions seen in the lungs of four patients we studied are consistent with a nonspecific response to acute lung injury that can be caused by infectious agents, trauma, drugs, or toxic chemicals. However, the multinucleated syncytial cells without viral inclusions seen in the lungs of two patients, including one patient positive by PCR, serologic, and virus-isolation methods, are suggestive of a number of viral infections, including measles and parainfluenzavirus, respiratory syncytial virus, and Nipah virus infection. Syncytia have occasionally been observed in culture cells inoculated with other group I and II coronaviruses that infect humans,[26-28] but they are more often described in culture cells infected with animal coronaviruses.[29-31] To our knowledge, syncytial cells have not been previously described in human tissues infected with coronaviruses.

We did not detect antigens of viruses associated with syncytial formation or SARS-associated coronavirus in these patient tissues, despite the severe pulmonary pathological processes. To detect this novel coronavirus antigen, we used an extensive panel of antibodies against coronaviruses representative of the three antigenic groups. The failure of these antiserum specimens to react with coronavirus antigens in the lung tissues of these patients could be attributed to clearance of viral antigens by the host immune response during the late stage of disease. The tissues of the four patients evaluated by immunohistochemical assays were obtained approximately two to three weeks into the course of the illness. For many viral infections, viral antigens and nucleic acids are cleared within two weeks of disease onset.[8,32] It is also possible that the pulmonary damage associated with SARS is not caused directly by the virus but represents a secondary effect of cytokines or other factors induced by viral infection proximal to but not within the lung tissue. In influenzavirus infections, viral antigens are seen predominantly in respiratory epithelial cells of large airways and are only rarely identified in pulmonary parenchyma, despite concomitant and occasionally severe interstitial pneumonitis.[33]

The clinical virology of this new disease is obviously in its infancy, and there is much to be learned about the behavior of the pathogen in the human host. For example, patterns of viral shedding, including timing and site, and the timing and characteristics of the host immune response, when defined, will help us understand disease transmission, infection-control practices, and strategies for developing vaccines. The CDC, in conjunction with other groups, is planning epidemiologic studies to address these and a wide range of other issues including risk factors for infection and severe disease and the efficacy of infection-control measures. These types of studies should help us refine and focus our efforts to control and treat SARS.

The investigation of the SARS outbreak serves as a positive template for laboratory and epidemiologic response to possible future infectious-disease pandemics. The rapid isolation and characterization of the novel coronavirus associated with SARS have allowed for the timely development of diagnostic tests that should aid our ability to understand the epidemiology of SARS and its prevention. Early recognition of this novel coronavirus has also made it possible to investigate antiviral compounds promptly and to begin developing vaccines. The speed with which this novel coronavirus was detected, characterized, and linked to SARS is a tribute to the power of the prompt communication and exchange of information among the World Health Organization collaborating laboratories about virus-isolation systems, PCR primers and virus sequences, and other diagnostic methods. This collaborative approach can be invaluable in our efforts to understand and control emerging public health threats.

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APPENDIX

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