

ORIGINAL ARTICLE

A Novel Coronavirus Associated with Severe Acute Respiratory Syndrome

Thomas G. Ksiazek, D.V.M., Ph.D., Dean Erdman, Dr. P.H., Cynthia Goldsmith, M.S., Sherif R. Zaki, M.D., Ph.D., Teresa Peret, Ph.D., Shannon Emery, Suxiang Tong, Ph.D., Carlo Urbani, M.D.,* James A. Comer, Ph.D., M.P.H., Wilina Lim, Pierre E. Rollin, M.D., Scott Dowell, M.D., M.P.H., Ai-Ee Ling, M.D., Charles Humphrey, Ph.D., Wun-Ju Shieh, M.D., Jeannette Guarner, M.D., Christopher D. Paddock, M.D., Paul Rota, Ph.D., Barry Fields, Ph.D., Joseph DeRisi, Ph.D., Jyh-Yuan Yang, Ph.D., Nancy Cox, Ph.D., James Hughes, M.D., James W. LeDuc, Ph.D., William Bellini, Ph.D., Larry J. Anderson, M.D., and the SARS Working Group†

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 six 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

No classic respiratory or bacterial respiratory pathogen was consistently identified. However, a novel coronavirus was isolated from patients who met the case definition of SARS. Cytopathological features were noted microscopically in Vero E6 cells inoculated with a throat-swab specimen. Electron-microscopical examination of cultures 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 fluorescent antibody tests and enzyme-linked immunosorbent assays made with the new coronavirus isolate have been used to demonstrate a virus-specific serologic response. Preliminary studies suggest that this virus may never before have infected 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. The name Urbani SARS-associated coronavirus is proposed for the virus.

From the Special Pathogens Branch (T.G.K., P.E.R., J.A.C.), Respiratory and Enteric Virus Branch (D.E., T.P., S.E., S.T., P.R., W.B., L.J.A.), Infectious Disease Pathology Activity (C.G., S.R.Z., C.H., W.J.S., J.G., C.P.), Influenza Branch (N.C.), and Office of the Director (J.W.L.), Division of Viral and Rickettsial Diseases, Division of Bacterial and Mycotic Diseases (S.D., B.F), and Office of the Director (J.H.), National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta; the World Health Organization, Hanoi, Vietnam (C.U.); the Department of Pathology, Singapore General Hospital (A.E.L.); DeRisi Laboratory, University of California, San Francisco (J.D.); the Centers for Disease Control, Department of Health, Taipei, Taiwan (J.-Y. Y.); the Government Virus Unit, Queen Mary Hospital, Hong Kong, China (W.L.); and the International Emerging Infectious Diseases Program, Bangkok, Thailand (S.D.).

*Deceased.

†Members of the SARS (Severe Acute Respiratory Syndrome) Working Group are listed in the Appendix.

This article was published at www.nejm.org on April 10, 2003.

Copyright © 2003 Massachusetts Medical Society.

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 late February 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 SARS case definition is available at <http://www.cdc.gov/ncidod/sars/>). 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, cell culture, electron microscopy, serology, 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 60Co 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 viral culturing is not 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, HELA, MDCK, HUT-292, 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 killed, 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 embed-

ding 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 $1000\times g$, 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 for use as an enzyme-linked immunosorbent assay (ELISA) antigen for antibody tests.

SEROLOGIC ANALYSIS

Spot slides were prepared by applying 15 μ l 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 and subsequent gamma irradiation of infected Vero E6 cells.⁷ The optimal dilution (1:1000) for the use of this antigen was determined by checkerboard titration against human serum from 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 in Singapore and Hong Kong and serum from healthy blood donors from the CDC serum bank and from persons infected with known human coronavirus (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 ob-

tained 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 μ m 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. Tissues from patients were also tested by immunohistochemical assays for various other viral and bacterial pulmonary pathogens.

Optimal dilutions of the primary antibodies were determined by titration experiments with coronavirus-infected cells from patients with SARS 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. In addition, a bronchoalveolar-lavage specimen was available from one patient for thin-section electron-microscopical evaluation.

RT-PCR

RNA extracts were prepared from 100 μ l 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 coronavirus 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 bron-

chitis 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'GCCTCTCTTGTCTTGTCTCGC3', and Cor-p-R1 (-) 5'CA-GGTAAGCGTAAACTCATC3', 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. 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 fluorescein (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 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 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.

SEQUENCING AND PHYLOGENETIC ANALYSIS

For sequencing, amplicons were purified with ExoSAP-IT (USB). Both strands of unlabeled products (or one strand of the 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).¹⁰ Phylogenetic trees were computed by maximum parsimony, distance, and maximum likelihood-based criteria analysis with PAUP (version 4.0.d10).¹¹

RESULTS

VIRUS ISOLATION

Two cell lines, Vero E6 cells and NCI-H292 cells, inoculated with oropharyngeal specimens from Patient 1, initially showed cytopathic effect. 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 further. The 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 E6 cells by thin-section electron microscopy revealed characteristic coronavirus particles within the cisternae of the rough endoplasmic reticulum and in vesicles (Fig. 2A).¹² 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.

The isolation and growth of a human-derived coronavirus in Vero E6 cells were unexpected. The known human coronaviruses are notably fastidi-

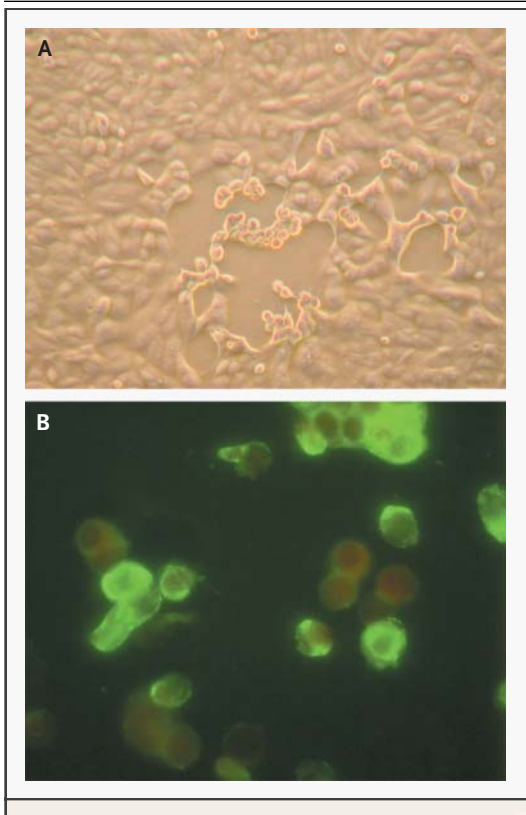


Figure 1. Vero E6 cells Inoculated with Oropharyngeal Specimens from Patients with SARS.

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

ous, preferring select cell lines, organ culture, or suckling mice for propagation. The only human or animal coronavirus that has been shown to grow in Vero cells is porcine epidemic diarrhea virus, and it requires the addition of trypsin to culture medium for growth in Vero E6 cells. Moreover, porcine epidemic diarrhea virus adapted to Vero cells results in a strikingly different cytopathic effect (i.e., cytoplasmic vacuoles and the formation of large syncytia).¹³ Syncytial cells were observed only occasionally in monolayers of Vero E6 cells infected with the new coronavirus; they clearly do not represent the dominant cytopathic effect.

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

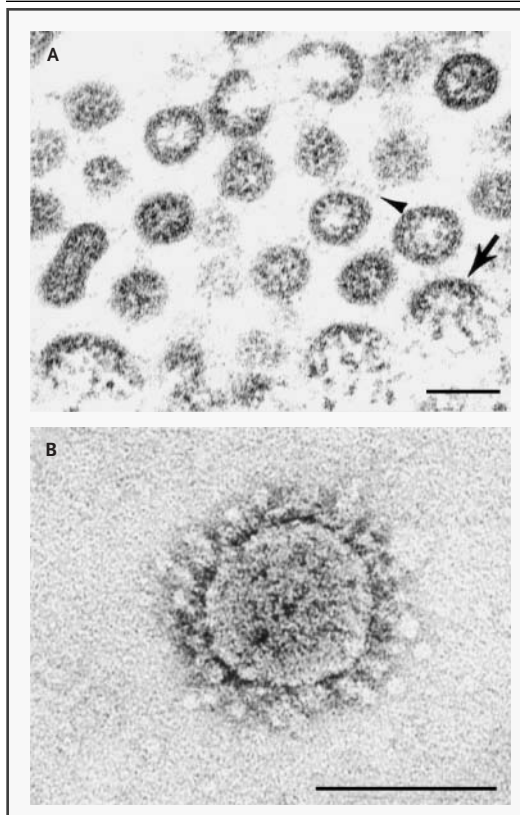


Figure 2. Ultrastructural characteristics of SARS-Associated Coronavirus Grown in Vero E6 cells.

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.

the broadly reactive primer set IN-2–IN-4. In contrast, this primer set produced no band against uninfected cells (the 229E and OC43 RNA controls).

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 nucleo-

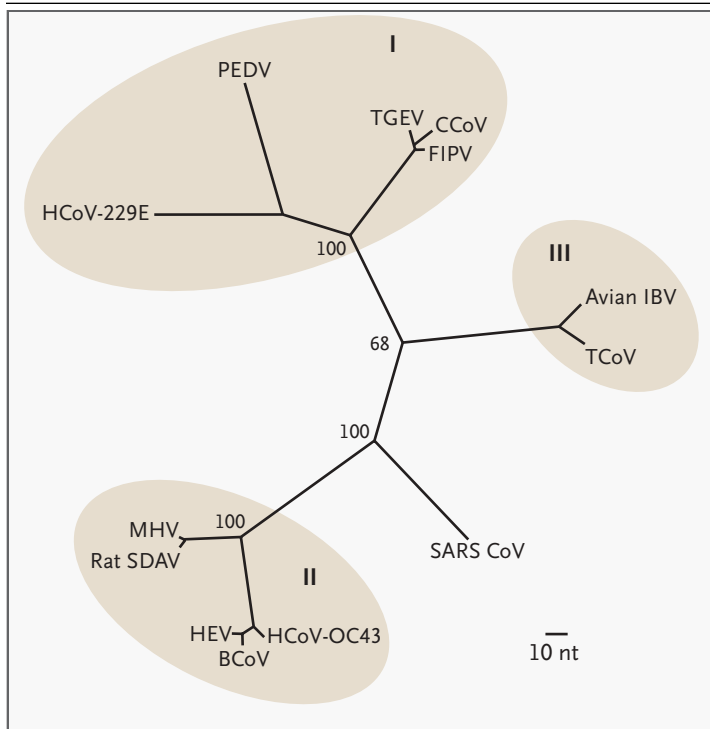


Figure 3. Estimated Maximum-Parsimony Tree Based on the Sequence Alignment of 405 Nucleotides of the Coronavirus Polymerase Gene Open Reading Frame 1b (Nucleotide Numbers 15173 to 15578 Based on Bovine Coronavirus Complete Genome Accession Number NC_003045) Comparing SARS Coronavirus with Other Human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

tide-sequence alignment is shown in Figure 3. Bootstrap analyses of the internal nodes at the internal branches of the tree provided strong evidence that the SARS-related 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 mem-

bers of the coronavirus family: avian infectious bronchitis and turkey coronavirus.¹⁴ Results were consistent with the identity of the isolate as a coronavirus.

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

Histopathological evaluation of lung tissues obtained from the autopsy of three patients and by open-lung biopsy in one patient 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 in two patients. 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 a patient with SARS, with the use of a battery of immunohistochemical stains reactive with coronavirus 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, metapneumoviruses, 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 viral cytopathic effect that included occasional multinucleated syncytial cells but no obvious viral inclusions (Fig. 4C). Immunohistochemical assays with various antibodies reactive with coronavirus 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 1). No staining was identified with any of several monoclonal or polyclonal antibodies reactive with

coronavirus in antigenic group II (human coronavirus OC43, bovine coronavirus, and mouse coronavirus) or group III (turkey coronavirus and infectious bronchitis virus). Electron-microscopical examination of a bronchoalveolar-lavage specimen 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 serum from patients with suspected SARS from Hong Kong, Bangkok, 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 2). Information from the limited numbers 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. Only 4 of the approximately 250 patients for whom samples were submitted for SARS testing were positive for coronavirus antibody. These four patients had a SARS-compatible illness and exposure to SARS through travel to affected regions. Moreover, 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 fluorescent 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 and Guangdong Province, China (Table 3).

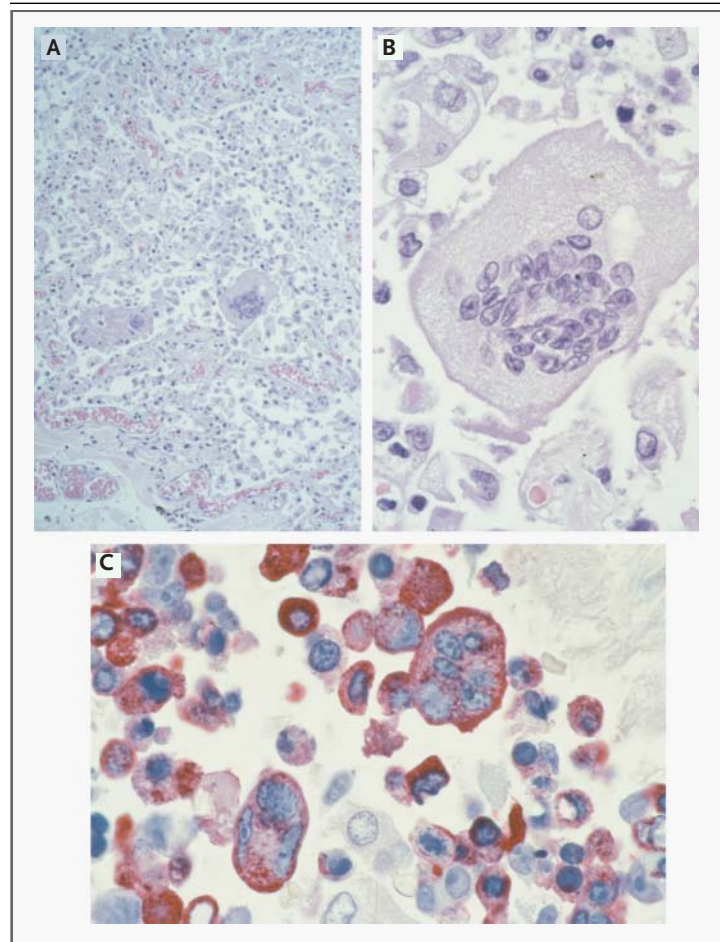


Figure 4. Histopathological Evaluation of Lung Tissue from Patients with SARS.

Tissue shows diffuse alveolar damage, abundant foamy macrophages, and multinucleated syncytial cells (Panel A). Higher magnification of syncytial cells show no conspicuous viral inclusions (Panel B). Panel C shows immunohistochemical staining of SARS-associated coronavirus-infected cells. Membranous and cytoplasmic immunostaining of individual and syncytial Vero E6 cells was undertaken with feline anti-feline infectious peritonitis virus 1 ascitic fluid. (Panels A and B, hematoxylin and eosin, $\times 50$ and $\times 250$, respectively; Panel C, immunoalkaline phosphatase with naphthol-fast red substrate and hematoxylin counterstain $\times 250$).

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 over the 405-nucleotide sequence from the coronavirus polymerase gene open reading frame 1b. For three 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, PCR, and serologic analysis; and for one by

Table 1. Immunohistochemical Reactivities of Various Polyclonal Group I Coronavirus Antibodies with a Coronavirus Isolated from a Patient with SARS.*

Antigen Target	Host	Immunohistochemical Reactivity with Coronavirus-Infected Culture Cells		
		SARS CoV (Vero E6)	HCoV-229E (mouse 3T3-hAPN)	FIPV-1 (BHK-fAPN)
SARS CoV	Human	+	+	-
HCoV-229E	Guinea pig, [†]	+	+	-
	Rabbit	+	+	+
FIPV-1	Cat	+	+	+
TGEV	Pig [‡]	+	-	+
	Pig [‡]	-	-	+

* 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.

[†] Reactivity was observed with two different polyclonal guinea pig antibodies.

[‡] Polyclonal porcine antibodies demonstrated different reactivity with the SARS-related CoV.

PCR and serologic analysis. We found none of the coronavirus-infected patients to be infected with human metapneumovirus. A variety of respiratory pathogens were also identified by RT-PCR in patients whose samples were submitted for SARS testing, including 5 with human metapneumovirus and 13 with a rhinovirus. None of the patients who were positive for human metapneumovirus had pneumonia. In only one patient was both SARS coronavirus and another respiratory virus detected; Patient 5 had both SARS coronavirus and a rhinovirus.

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 point to a possible 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. This approach shows the power of a global collaborative effort to address the threat of emerging infectious diseases.¹⁵

The identification of this novel coronavirus relied on classic tissue-culture isolation to amplify the pathogen and then on electron-microscopical

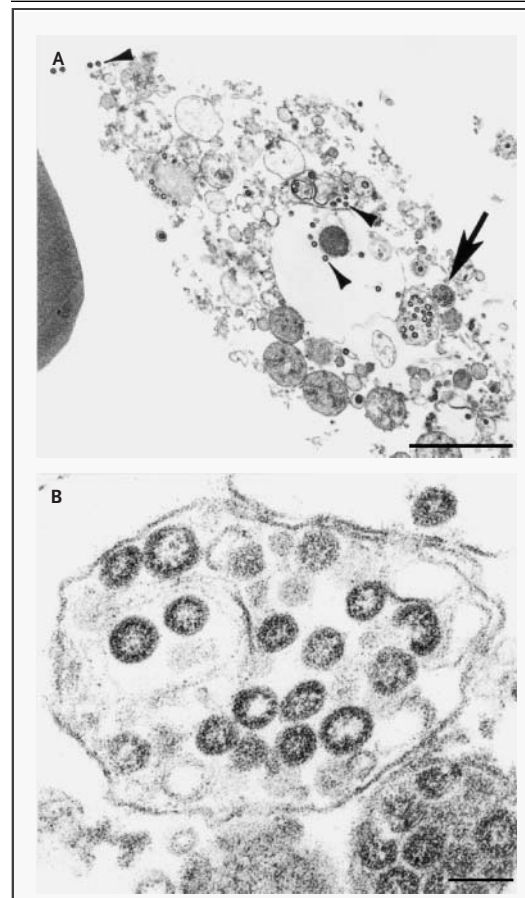


Figure 5. Ultrastructural Characteristics of a Coronavirus-Infected Cell in Bronchioalveolar-Lavage Fluid from a Patient with SARS, Showing Numerous Intracellular and Extracellular Particles.

The particles 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 100 nm, and that in Panel B, 1 μ m.

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 prior knowledge or specific reagents but that could nevertheless categorize a pathogen on the basis of its appearance and morphogenetic features.¹⁶⁻¹⁹

In this report, we describe infection in 18 pa-

Table 2. 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 C.

Source	Serum No.	Days after Onset	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	4	6400	400
Singapore	4.1	5	100	<25
Singapore	4.2	16	1600	400

* The value is the reciprocal of the dilution.

Table 3. Specimens from Patients with SARS That Were Positive for SARS-associated Coronavirus by One or More Methods.

Patient No.	Exposure	Serologic Results	Specimen	Isolation	PCR
31*	Hong Kong	Positive	Serum	Negative	Not done
39	Hong Kong	Positive	Serum	Negative	Negative
94*	Hong Kong	Positive	Serum	Negative	Negative
220	Hong Kong	Not done	Sputum	Positive	Positive
0	Hong Kong	Positive	Kidney, lung, bronchoalveolar lavage	Positive	Positive
1	Vietnam	Negative	Throat wash	Positive	Positive
3	Vietnam	Negative	Throat wash	Negative	Positive
8	Vietnam	Negative	Throat wash	Negative	Positive
10	Vietnam	Negative	Throat wash	Negative	Positive
13	Vietnam	Negative	Throat wash	Negative	Positive
16	Vietnam	Negative	Throat wash	Negative	Positive
17	Vietnam	Negative	Throat wash	Positive	Positive
20	Vietnam	Negative	Throat wash	Negative	Positive
26	Vietnam	Negative	Throat wash	Negative	Positive
77	Vietnam	Positive	Nasal and throat swab	Positive	Positive
78	Canada	Not done	Lung, bone marrow	Negative	Positive
79	Taiwan	Negative	Sputum	Negative	Positive
80	Hong Kong	Positive	Oropharynx, serum	Negative	Positive

* This was a late specimen, antibody positive at first sample.

tients with SARS who were epidemiologically linked either to the source of the Hong Kong cases 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. A coronavirus with identical sequences has also been detected in a patient with SARS in Canada.⁵ The virus was found in multiple specimens including extracts of lung and kidney tissue by virus isolation or PCR; bronchoalveolar-lavage specimens by electron microscopy and PCR; and sputum or upper respiratory tract swab, aspirate, or wash specimens by PCR or isolation. Although we tested specimens from the coronavirus-positive patients for a variety of other respiratory pathogens, including human metapneumovirus, by PCR, none were detected in these coronavirus-positive patients except for a rhinovirus in Patient 1. 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 tissue disease. We were, however, not able to demonstrate the coronavirus antigens or RNA in cells of disease tissue histologically or to demonstrate a direct involvement in the pathologic process. Neither were we able to demonstrate SARS-related coronavirus infection in all patients with SARS.

Possible reasons for the inability to demonstrate infection in some patients with SARS include lack of sufficient sensitivity of the assays to detect the pathogen or the immune response and the timing and type of specimens tested. For example, we have received convalescent-phase serum specimens from many patients with suspected SARS and have serologically ruled out infection in many such patients. In addition, we are just beginning to study the type and timing of clinical specimens that will be 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 our ability to detect this virus and its footprints. In addition, the case definition of SARS is very broad and is likely to include infections by other agents. We are also continuing to examine 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 the patient with laboratory-confirmed SARS will certainly help to narrow the case definition.

The apparent lack of antibody in all serum specimens except those from patients with SARS suggests that this virus has not previously circulated. Certainly, it has not circulated widely in humans, which is further evidence in favor of the association between infection with this novel coronavirus and SARS. Because of the death of Dr. Carolo Urbani during the investigation of the initial SARS epidemic, we propose that the virus be named Urbani SARS-associated coronavirus. 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 on this coronavirus suggest that it is sufficiently distinct from those previously reported in animals and humans that its source may be yet to be discovered. Interestingly, no other human coronavirus and only one animal coronavirus,

recently isolated in China from pigs with respiratory disease,¹³ has been noted to replicate in Vero cells. The sequences of this porcine virus are distinct from those of the SARS coronavirus and indicate that this virus is not the parent virus of SARS coronavirus.

The three known groups of coronavirus are associated with a variety of diseases in 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 to cause severe disease in animals 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 SARS-related coronavirus may be the first example of a coronavirus that causes severe disease in humans.

Pathological studies of patients who died with SARS show diffuse alveolar damage in the lung 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 the four patients we studied are consistent with a nonspecific acute response to lung injury that can be caused by infections, trauma, drugs, or toxic chemicals. The multinucleated syncytial cells without viral inclusions seen in the lungs of two patients, however, are suggestive of a number of viral infections including measles and parainfluenzavirus, respiratory syncytial virus, and Nipah virus infection. Multinucleated syncytial cells associated with some human coronavirus infections have occasionally been observed in cell culture,^{20,21} but most often in cell cultures inoculated with animal coronaviruses.²²⁻²⁴ To our knowledge, syncytial cells have not been described previously in human tissues infected with coronavirus.

We did not detect antigens of virus associated with syncytial formation or Urbani SARS-associated coronavirus in these tissues, despite the severe

pulmonary pathologic processes. To detect this novel coronavirus antigen, we used an extensive panel of antibodies against coronaviruses that are representative of the three antigenic groups, including several group 1 antiserum specimens that reacted against Urbani SARS-associated coronavirus-infected tissue-culture material. A possible explanation for the failure of this antiserum to react with antigens in these patients on immunohistochemical analysis is that the host immune response had cleared the virus from these tissues. The tissues were available late in the course of the illness, 14 to 20 days after its onset. For many viral respiratory infections, viral antigens and nucleic acids are cleared within two weeks after the onset of disease.^{8,25} It is also possible that the tissue damage in SARS is not directly related to viral infection in tissues but is a secondary effect of cytokines or other factors induced by viral infection proximal to but not within the lung tissue. In influenza infections, viral antigens are seen predominantly in respiratory epithelium of large airways and are only rarely identified in pulmonary parenchyma, despite concomitant and occasionally severe interstitial pneumonitis.²⁶

The investigation of the SARS outbreak serves as a model for laboratory and epidemiologic responses to possible future pandemics of infectious disease. The rapid isolation, characterization, and recognition of the etiologic agent in this outbreak have allowed for the rapid formulation of diagnostic tests, which should aid in understanding the epidemiology of SARS and its prevention. Early recognition of the etiologic agent also has made the virus available for rapid investigation of antiviral compounds and vaccines. In addition, there has been prompt communication among scientific teams on multiple continents. The exchange of valuable information on viral-isolation systems, PCR primers and resulting virus sequences, and diagnostic approaches has led to rapid progress in many laboratories toward identifying the etiologic agent of the SARS outbreak.

APPENDIX

Members of the SARS Working Group include the following: D. L. Cannon, M. Curtis*, B. Farrar, L. Morgan, L. Pezzanite*, A. Sanchez, K.A. Slaughter, T.L. Stevens, P.C. Stockton, K.D. Wagoner, A. Sanchez, S. Nichol, M. Vincent, J. Osborne, J. Honig, B. (Special Pathogens Branch, Division of Viral and Rickettsial Diseases); B. Holloway, K. McCaustland (Special Pathogens Branch, National Center for Infectious Diseases); J. Lingappa, L. Lowe, S. Scott, X. Lu, Y. Villamarzo, B. Cook, C. Birge, B. Shu, M. Pallansch (Respiratory and Enteric Virus Branch, Division of Viral and Rickettsial Diseases); K.M. Tatti, T. Morken, C. Smith, P. Greer, T. McGlothen, J. Bhatnagar, M. Patel, J. Bartlett, J. Montague, W. Lee, M. Packard (Infectious Diseases Pathology Activity, Division of Viral and Rickettsial Diseases); A. Moen, K. Fukuda, T. Uyeki, S. Harper, A. Klimov, S. Lindstrom (Influenza Branch, Division of Viral and Rickettsial Diseases); R. Benson, G. Carlone, R. Facklam, P. Fields, P. Levett, L. Mayer, D. Talkington, W.L. Thacker, M.L.C. Tondella, A. Whitney (Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases); B. Robertson, D. Warnock (SARS Laboratory Team); S. Schrag (SARS State /Domestic Team); D. Ganem, University of California, San Francisco; S.M. Poutanen, Department of Laboratory Medicine and Pathobiology, University of Toronto; T.-J.

Chen, M.D., M.P.H., Center for Disease Control, Department of Health, Taiwan; C.-H. Hsiao, Department of Pathology, National Taiwan University Hospital, Taipei, Taiwan; N.G. Wai-fu, Yan Chai Hospital, Hong Kong; M. Ho, K. Wah Hospital, Hong Kong; Ng Tak-keung, Princess Margaret Hospital, Hong Kong; and the WHO SARS Collaborating Laboratory Network: Centers for Disease Control and Prevention, Atlanta; Public Health Laboratory Service, Central Public Health Laboratory, London; Government Virus Unit, Hong Kong; Prince of Wales Hospital, Chinese University of Hong Kong, Hong Kong; Queen Mary Hospital, Hong Kong; Centers for Disease Control, Beijing, China; Erasmus Universiteit, National Influenza Center, Rotterdam, the Netherlands; Federal Laboratories for Health Canada, Winnipeg, Canada; Bernhard Nocht Institute, Hamburg, Germany; Institut für Virologie, Marburg, Germany; and National Institute of Infectious Disease, Tokyo, Japan. (*detailed)

We are indebted to Ann R. Falsey and Edward E. Walsh, Rochester General Hospital and the University of Rochester School of Medicine and Dentistry, Rochester, New York; Kay Holmes, University of Colorado Health Sciences Center, Denver; University of California, San Francisco, and Linda Saif, Ohio State University; to John Black, James Guy, Miladin Kostovic, Julia Ridpath, Joan Beck, Edward Dubovi, Sanjay Kapil, Chris Grant, David Swayne, Julian Leibowitz, and S.A. Naqi for the provision of reference serum, monoclonal antibodies, and coronavirus control tissues; and to the many private and public health physicians and laboratorians who treated patients with SARS or provided clinical materials for evaluation for their assistance and cooperation. This article is dedicated to the memory of Carlo Urbani.

REFERENCES

1. Acute respiratory syndrome in China — update 3: disease outbreak reported: Geneva: World Health Organization, February 2003.
2. Update: outbreak of severe acute respiratory syndrome — Worldwide, 2003. *MMWR Morb Mort Wkly Rep* 2003;52:241-8.
3. Tsang KW, Ho PL, Ooi GC, et al. A cluster of cases of severe acute respiratory syndrome in Hong Kong. *N Engl J Med* 2003;348:xxxx-xx.
4. Lee N, Hui DH, Wu A, et al. A major outbreak of severe acute respiratory syndrome in Hong Kong. *N Engl J Med* 2003;348:xxxx-xx.
5. Poutanen SM, Low DE, Henry B, et al. Identification of severe acute respiratory syndrome in Canada. *N Engl J Med* 2003;348:xxxx-xx.
6. Wulff H, Lange JV. Indirect immunofluorescence for the diagnosis of Lassa fever infection. *Bull World Health Organization* 1975;52:429-36.
7. Ksiazek TG, West CP, Rollin PE, Jahrling PB, Peters CJ. ELISA for the detection of antibodies to Ebola viruses. *J Infect Dis* 1999;179:(Suppl 1):S192-S198.
8. Zaki S, Greer PW, Coffield LM, et al. Hantavirus pulmonary syndrome: pathogenesis of an emerging infectious disease. *Amer J Pathol* 1995;146:552-79.
9. Falsey AR, Erdman D, Anderson LJ, Walsh EE. Human metapneumovirus infections in young and elderly adults. *J Infect Dis* 2003;87:785-90.
10. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994;22:4673-80.
11. Swofford DL. PAUP 4.0: phylogenetic analysis using parsimony (and other methods). Sunderland, Mass.: Sinauer, 1999.
12. Oshiro LS, Schieble JH, Lennette EH. Electron microscopic studies of coronavirus. *J Gen Virol* 1971;12:161-8.
13. Hofmann M, Wyler R. Propagation of the virus of porcine epidemic diarrhea in cell culture. *J Clin Microbiol* 1988;26:2235-9.
14. Jonassen CM, Jonassen TO, Grinde B. A common RNA motif in the 3' end of the genomes of astroviruses, avian infectious bronchitis virus and an equine rhinovirus. *J Gen Virol* 1998;79:715-8.
15. Smolinski MS, Hamburg MA, Lederberg J, eds. *Microbial threats to health: emergence, detection, and response*. Washington, D.C.: National Academies Press, 2003.
16. Hazelton PR, Gelderblom HR. Electron microscopy for rapid diagnosis of infectious agents in emergent situations. *Emerg Infect Dis* 2003;9:294-303.
17. Johnson KM, Lange JV, Webb PA, Murphy FA. Isolation and partial characterization of a new virus causing acute haemorrhagic fever in Zaire. *Lancet* 1977;1:569-71.
18. Chua KB, Bellini WJ, Rota PA, et al. Nipah virus: a recently emergent deadly paramyxovirus. *Science* 2000;288:1432-5.
19. Murray K, Selleck P, Hooper P, et al. A morbillivirus that caused fatal disease in horses and humans. *Science* 1995;268:94-7.
20. Patterson S, Macnaughton MR. Replication of human respiratory coronavirus strain 229E in human macrophages. *J Gen Virol* 1982;60:307-14.
21. Luby JP, Clinton R, Kurtz S. Adaptation of human enteric coronavirus to growth in cell lines. *J Clin Virol* 1999;12:43-51.
22. Lavi E, Wang Q, Weiss SR, Gonatas NK. Syncytia formation induced by coronavirus infection is associated with fragmentation and rearrangement of the Golgi apparatus. *Virology* 1996;221:325-34.
23. Kusanagi K, Kuwahara H, Katoh T, et al. Isolation and serial propagation of porcine epidemic diarrhea virus in cell cultures and partial characterization of the isolate. *J Vet Med Sci* 1992;54:313-8.
24. Jacobse-Geels HE, Horzinek MC. Expression of feline infectious peritonitis coronavirus antigens on the surface of feline macrophage-like cells. *J Gen Virol* 1983;64:1859-66.
25. Wong KT, Shieh W-J, Kumar S, et al. Nipah virus infection: pathology and pathogenesis of an emerging paramyxoviral zoonosis. *Am J Pathol* 2002;161:2153-67.
26. Guarner J, Shieh WJ, Dawson J, et al. Immunohistochemical and in situ hybridization studies of influenza A virus infection in human lungs. *Am J Clin Pathol* 2000;114:227-33.

Copyright © 2003 Massachusetts Medical Society.