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Molecular Biological Analysis of SARS Viruses in Taiwan

Introduction

The first case of atypical pneumonia (SARS as it was called initially) was an American businessman who became ill on 26 February 2003 in Hanoi. He was later sent to Hong Kong for treatment and died there. Thereafter, there had been in Hong Kong and Vietnam cases of atypical pneumonia complicated with respiratory failure. By late February of the year, there had been in Guangdong Province of China 305 cases of atypical pneumonia with five deaths. Taiwan was, unfortunately hit by the infection in mid-March. A businessman from Taiwan, upon his return from a trip to Guangdong Province, developed symptoms of fever, short breath, and pneumonia, and was admitted to the National Taiwan University Hospital for medical care. There have also been cases in Canada, Singapore and the US. Some medical personnel are infected while caring for patients. The pathogenic agent was initially suspected to be Chlamyida pneumoniae. The infection features infiltrate pneumonia and respiration failure, and is more serious than the atypical pneumonia caused by some known viruses or bacteria. The epidemic has attracted attention and research efforts of many scientists around the world.

For distinction and definition, the World Health Organization, on 15 March, named the new infection “severe acute respiratory syndrome, SARS”(1).

Management

In the early stage of the SARS outbreak in March, all viruses and bacteria that are likely to induce atypical pneumonia were suspected. Upon receipt of the suspected SARS specimens, the Virology Laboratory of the Laboratory Testing Division immediately conducted laboratory analysis of all pathogenic agents likely to induce short breath such as tuberculosis bacilli, chlamydia, influenza virus, avian flu virus (H5,H7,H9), parainfluenza virus, measles virus, mumps virus, adenovirus, respiratory syncytial virus, Nipah virus, Hendra virus, and Hanta virus, with all showing negative.

The epidemiologist of the US CDC stationed in Thailand and his colleagues then arrived in Taiwan to help in the investigation. A large number of people from Taiwan visit the mainland China each year for trade, tourism and meeting relatives. The Department of Health, to understand the source of infection for disease control and to prevent the spread of the infection, sent some suspected SARS specimens to the US CDC in Atlanta for laboratory testing. At the same time, the Chinese University of Hong Kong announced that they had succeeded in identifying and culturing the SARS pathogen, which they believed was human metapneumovirus (hMPV) of the paramyxoviridae family. The lung tissues of SARS patients pathological changes indicate the likelihood of viral infection. However, the US CDC has failed to identify any hMPV from all suspected SARS specimens around the world.

Later, based on the information supplied by the US CDC, the Virology Laboratory began to use both the conventional and molecular biological methods for the analysis of coronavirus.

Specimen Collection and Laboratory Testing

Biosafety

SARS is classified by the Department of Health as a Category IV notifiable disease. Pathogenic agents of diseases under category BL4 are defined as those that are highly dangerous to individuals and members of the community, are likely to cause serious diseases in men or animals, and can be transmitted from patients to others. SARS specimens, therefore, by regulations on the isolation facilities of biologically-safe laboratory, should be handled in P2 and above laboratories, and P3 and above laboratories for the isolation and culture of SARS viruses. Laboratory technicians should be trained in advance and equipped with basic biological-safety knowledge in the sterilization of laboratory equipment and disposal of wastes. While in operation, they should wear isolation facilities to protect themselves. Biological wastes should be chemically or physically disinfected and sterilized.

Sources of Specimens

Specimens could be collected from all patients meeting the definitions of "suspected cases" or "reportable cases" of medical centers, teaching hospitals and clinic. A suspected case is one who shows a temperature of higher than 38℃ and symptoms of upper respiratory tract infection such as coughing, short breath or dyspnea, or infiltrate pneumonia in x-ray examination(2). Cases could be reported with or without history of traveling or contact. Cases meeting the definition of suspected cases and confirmed by x-ray as pneumonia or showing symptoms of short breath should be taken their upper respiratory tract specimens such as throat swabs, saliva, sputum, blood, feces, urine and sent at low temperature to the Virology Laboratory for virus assessment.

Pre-processing of Specimens

1) Blood Specimens: serum or plasma added sodium citrate or EDTA could be used. Specimens were transported at 4℃. Specimens were centrifuged in 2,000 rpm for 10 minutes, and the serum thus isolated was used. Specimens were named and coded. Serum was put in two tubes, one at 4℃ for RT-PCR, and one kept at -20℃.

2) Throat Swabs: stirred with cotton swab, squeezed and removed. The fluid was kept at 4℃, centrifuged at 2,100 xg for 15 minutes. The upper fluid was collected, placed in tubes, named and numbered, and kept at -70℃.

Cell culture tubes inoculated with specimens were centrifuged at 3000 rpm for 15 minutes to collect the upper clear fluid. The sediment cells suspected of infection were treated with indirect immunofluorescence assay (IFA) and observed under florescence microscopes. When there was apple green fluorescence on the cytoplasm, it was read influenza virus positive.

3) Fecal Specimens: 1 gram of feces was placed in centrifugation tube, added glass balls and 10 ml of PBS to make a 10% suspension, placed at 4℃, and centrifuged at 2,100 xg for 15 minutes. The upper fluid was removed to centrifugation tubes, added by its volume 1/10 of Chloroform, shaken and mixed for 15 minutes, placed at 4℃, and centrifuged at 2,100 xg for 15 minutes. The upper fluid was placed in 2-3 cryotubes, numbered and dated, and kept at -70℃.

4) Sputum Specimens: mixed with 0.9% NaCl (containing 1% N-Acetylcysteine) by 1:2, stirred and placed for 30 minutes at 4℃, centrifuged at 2,100 xg for 15 minutes. The upper fluid was collected.

Notes: Errors could easily occur in specimen collection. The amount collect was insufficient, or the specimens contained only oral, nasal or pharyngeal discharges but sputum. Before collection, teeth should be brushed. Sputum

then was coughed out of the respiratory tract. The sputum specimens collected should be discharges of the lungs. More sputum was found in early morning. Sputum thus collected should be kept in sealed, sterilized plastic container to prevent it from infecting the collector or others.

Assessment of SARS Viruses

Extracting RNA

RNA was purified by the QIAmp Viral RNA kit of QIAGEN. 140 μl of saliva and pharyngeal swabs and blood of the patients were collected, added 560 μl of buffer AVL, and placed under room temperature for 10 minutes, added again 560 μl of pure alcohol for vortexing. The mixture was put through QIAmp spin column. The column was washed with buffer AW twice, and dissolved RNA with 80℃ pure water. Viruses thus prepared could be used for reverse transcription polymerase chain reaction.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

1) Reverse Transcription

10 μl of virus RNA was added in 50 pmole mixture of 75 mM KCl, 50 mM Tris-HCl, 3 mM MgCl2, 10 mM DTT, 0.5 mM ATCG dNTP mixture, RNasin 38U/μl, and antisense primer IN4 or IN7 (Table 1)(4), kept at 70℃ for 10 minutes, added again 100 units of supercript II-reverse transcriptase, and kept at 37℃ for 90 minutes.

2) PCR

(1) First round PCR

The cDNA collected from the reverse transcription was used for PCR. The cDNA was added 50 mM KCl, 10 mM Trais-HCl, 1.5 mM MgCl2, 0.1% Triton-X 100, ATCG dNTP mixture 1 mM, and primer In2/In4 or In6/In7 mixture of 50 pmole each, added 5 units of Taq polymerase (Invitrogen), denatured at 94℃ for 3 minutes, repeated reactions 35 times at 94℃ for 30

seconds, 50℃ for 45 seconds, and 72℃ for one minute, and finally at 72℃ for 10 minutes.

(2) Nest PCR

5 μl of the product of the first-round PCR was added 50 mM KCl, 10 mM Tris-Cl, 1.5 mM MgCl2, 0.1% Triton-X 100, ATCG dNTP mixture 1 mM, and F2/R1 or F3/R1 of 50 pmole each, added again 5 units Taq polymerase (Promega), denatured at 94℃ for 3 minutes, and repeated reactions for 35 times at 94℃ for 30 seconds, 55℃ for 45 seconds, 72℃ for one minute, and finally at 72℃ for 15 minutes.

3) Sequencing

ABI PRISM™, BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) were used to mark the nucleic acid products to be analyzed. The purity of nucleic acid may affect the quality of sequencing, nucleic acid products of high purity (OD260/280>1.8) were used as sequencing models. The amount of nucleic acid needed was 200-500 ng for double-ply DNA , and 50-100 ng for single-ply DNA and 30-90 ng PCR products. Some adequate amount of nucleic acid models, 3 μl premix (containing Tris-HCl buffer, pH 9.0, MgCl2, dNTP mix, labeled A-dye terminator, C-dye terminator, G-dye terminator, T-dye terminator, AmpliTag DNA polymerase FS with thermally stable pyrophosphatase), 3.2-5.0 pmole nucleic acid primers (2-Rabies-F/2-Rabies-R was used in the present study), were mixed equally with water to a total volume of 10 μl. The mixture was then covered with some mineral oil. The centrifugal tube containing the product was placed in PCR reactor preheated to 94℃. Reactions were repeated 25 times at 94℃ for 30 seconds, 55℃ for 15 seconds, 60℃ for 4 minutes, and finally at 4℃.

Purification of Product – Alcohol Sedimentation

To reduce interference to the signal reading by some free dye terminator, the sequenced products should be purified further for nucleic acid sequencing. There were many ways to purify sequenced products, and the present study used the alcohol sedimentation method. The marked product was first sucked, removed excessive paraffin oil with paraffin paper, and placed in centrifugal tubes, added 2 μl 3 M sodium acetate (NaOAc, pH 4.6) and 50 μl 95% ethanol, and mixed thoroughly. The mixture was placed under room temperature for 15 minutes, centrifuged at 15,000 g for 30 minutes. The upper liquid was removed carefully, and the sediment was washed with 250 μl of 70% ethanol. After some shaking, the mixture was centrifuged at 15,000 g for 10 minutes, and dried with vacuum drier, kept at -20℃. It was dissolved again in 3 μl loading buffer (deionized formamide: 25 mM EDTA, pH 8.0 = 5:1).

The DNA section augmented by RT-PCR was purified with alcohol for sequencing with DNA autosequencer.

Analysis of Phylogenetic Tree

The known coronavirus sequences such as human coronavirus (229E), bovine coronavirus (BcoV), canine coronavirus (CcoV), feline infectious peritonitis virus (FIPV), human coronavirus (HcoV), avian infectious bronchitis virus (IBV-A), mouse hepatitis virus (MHV), porcine hemagglutinating encephalomyelitis virus (PHEV), rat sialodacryoadenitis virus (RAT-SDAV), turkey coronavirus (TcoV), porcine transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhea virus (PEDV), CH-TW (serial numbers in gene bank are AF124992, Z34093, AF124987, AF124986, NC002645, NC001451, AF124991, M55148, NC001486, AF124990, AF124989, AF124988, AF124985, NC003045, AY268049) and virus sequences collected from patients were analyzed for their phylogenetic trees. The procedures were operated with the

molecular evolutionary genetics analysis (MEGA) version 2.1. The "neighbor-joining" method was used and bootstrapped for 1,000 times.

Results

Suspected SARS specimens extracted RNA were analyzed with primers by RT-PCR and nest-PCR. It was found that after augmentation in the first-round RT-PCR, in many specimens 405bps sections were not found by electrophoresis analysis. Only about 368bps sections could be seen by electrophoresis analysis after further augmentation by nest-PCR (Figure 1). The nest-PCR products, after purification, were sequences by AB1377. The sequences thus obtained were compared with the NCBI on the Internet to find only 58 nucleotides were similar to human coronaviruses, the rest sequences were not the same.

When different specimens were augmented by RT-PCR and nest-PCR and electrophoresis analyzed with agar, it was found that throat swabs and sputum were the best specimens to detect coronavirus sequences. Coronavirus sequences could also be detected from sera collected at the early stage of onset. In a few fecal specimens, coronavirus was detected. No coronaviruses were detected in the rest specimens (data not shown).

Virus sequences CH-TW obtained from the pol genome region of specimens augmented by RT-PCR and nest-PCR (the first suspected SARS case in Taiwan, a businessman surnamed Chin stationed on the mainland China, his throat swabs were augmented with RT-PCR, and the assession number was AT268049), and the known coronaviruses (see Materials and Method) were compared for similarity in nucleotide and amino acid to find that CH-TW and other known coronaviruses were 54%-62% similar in nucleotide. Of them, MHV-NC-001846 (similarity 62.9%), MHV-M55148

(62.4%), and RAT-SDAV-AF124990 (62.1%) were most similar to the CH-TW; and CcoV-AF124986 (55%) was the least similar. The three genome sequences of higher similarity, their viruses were isolated from rats; the one of least similarity, the virus came from dogs (Table 2).

By similarity in amino acid sequences, it was found that the similarity between CH-TW and other known coronaviruses was between 55% and 73%. The coronavirus isolated from pigs, PHEV-AF1249880, had the highest amino acid similarity of 73.1%; the virus isolated from humans, HcoV-OC43-AF124989, and the viruses isolated from cows, BcoV-AF124985 and BcoV-NC-003045, had 72.3% similarity; and the viruses isolated from pigs, TGEVP-AF124992 and TGEV-Z34093, had a similarity of only 55.4% (Table 3).

The CH-TW genome sequence and other known coronaviruses were analyzed for their phylogenetic trees. 390bp each was extracted from sequences, processed with Molecular Evolutionary Genetics Analysis (MEGA) version 2.1 by “neighbor-joining” method, and bootstrapped 1,000 times. It was found that the phylogenetic trees came in three clusters (Figure 2) of group I, group II and group III. For larger difference, the CH-TW was a separate branch of itself(3). By phylogenetic tree analysis, it could be speculated that the CH-TW was a mutated coronavirus strain, and was significantly different from the coronaviruses that have been isolated from humans, pigs, cows and dogs.

Discussion and Conclusion

A significant difference in sequences exists between this novel coronavirus and other already detected coronaviruses. At the beginning, when the Hong Kong University reported to have observed virus of the paramyxoviridae family

through electronic microscopes, research was led to this direction. The Virology Laboratory of this Division had also attempted to isolate viruses from suspected specimens collected in Taiwan by all methods and techniques without much success. Then the US CDC came up with some discovery from part of the inoculated cells (Vero-E6). They noticed from Vero-E6 some changes (CPE)(4), some crown-like viruses (Figure 3). Coronavirus is one of the pathogenic agents of common cold, and detection of coronavirus from specimens is not uncommon. When BAL (Broncheoalveolar lavage) was directly observed with electronic microscopes, coronavirus similar to the one observed was also noticed. It was then speculated from these facts that coronavirus was the pathogenic agent of this epidemic. Later, more laboratories around the world had also detected this novel coronavirus from specimens of suspected cases(5). On 16 April, the WHO officially announced that coronavirus of the mutated coronaviridae family was the pathogenic agent of SARS.

In the course of the epidemic, conventional detection methods for viruses had functioned in full. Starting with the discovery of coronavirus-like virus by the US CDC, research began to focus on this direction. With the advancement in molecular biology technologies and efforts of scientists around the world, the genome sequences of SARS virus were finally decided. The length of the gene was between 29000 and 31000 nucleotides(6). The genome sequences announced by Canada and the US CDC were 29736 and 29727 nucleotides respectively. The virus sequences announced by the Hong Kong University and the Chinese University of Hong Kong were 29742 and 29702 nucleotides respectively, similar to those found in Canada and the US. The research team of the National Taiwan University Hospital had also sequenced indigenous SARS. The Virology Laboratory of the Center for Disease Control is in the

process of sequencing the length of viruses isolated from deceased patients of SARS.

In the phylogenetic tree analysis, though SARS viruses could not be placed under the three known coronavirus groups, by comparing nucleotides, it was noticed that the SARS viruses were higher in similarity with the middle sequence of the murine hepatitis virus of the second group, and the mid-post sequence of the avian infectious bronchitis virus of the third group(4). The Virology Laboratory will attempt to, based on the already discovered genome sequences, understand the mutation rate of the virus to try to develop diagnostic tools for SARS. Thus far, a real-time RT-PCR fluorescent detection system has been developed. This method of high rapidity, sensitivity and specificity can mass screen a large number of specimens at one time. The IFA and EIA are currently under testing. Laboratory diagnosis is the front line of disease control to early report and predict disease situation, to timely conduct disease surveillance, and to early prevent the spread of diseases. For the effective control of SARS, rapid and accurate laboratory testing methods are most essential.

The Department of Health announced on the evening of 27 March SARS a notifiable disease of Category IV. The WHO further listed Taiwan as an affected area(9). The Center for Disease Control was from the beginning fully engaged in disease control and laboratory diagnosis, in the surveillance of the development of the epidemic, and in taking necessary control measures to minimize the impact of SARS on the health of the population. Thus far, scientists have not yet developed effective measures against SARS. The public should be urged to avoid as much as possible traveling to affected areas, visiting to public places, and be more self-protective.

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Table 1 Primers needed for RT-PCR and nest-PCR are positive ply to S (sense), and negative to AS (anti-sense).

Primers	Sequence
IN2-S	5’-GGGTTGGGACTATCCTAAGTGTGA-3’
IN4-AS	5’-TAACACACAAACACCATCATCA-3’
IN6-S	5’-GGTTGGGACTATCCTAAGTGTGA-3’
IN7-AS	5’-CCATCATCAGATAGAATCATCATA-3’
F2-S	5’-CTAACATGCTTAGGATAATGG-3’
F3-S	5’-GCCTCTCTTGTTCTTGCTCGC-3’
R1-AS	5’-CAGGTA AGCGTAAAACTCATC-3’

Table 2

Percent Similarity

Percent Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
1		57.5	61.1	61.6	55.0	55.8	60.9	58.7	62.4	62.9	61.4	62.1	58.2	55.8	55.8	60.1	1
2	62.9		58.6	58.6	67.8	69.1	59.1	55.6	58.6	58.6	58.6	58.3	56.4	68.5	68.5	69.3	2
3	55.0	60.3		99.5	58.3	58.1	96.2	58.7	82.9	82.6	98.2	83.6	58.7	57.5	57.5	53.2	3
4	53.9	60.3	0.5		58.6	58.3	95.9	59.0	82.6	82.4	98.0	83.4	59.0	57.8	57.8	52.7	4
5	69.4	42.4	60.9	60.4		95.7	57.5	55.9	59.6	59.6	58.3	59.8	54.6	95.7	95.7	65.0	5
6	67.3	40.1	61.5	60.9	4.5		56.8	56.4	59.8	59.8	58.1	60.1	55.4	96.7	96.7	65.7	6
7	55.5	59.2	3.9	4.2	62.8	64.6		59.7	82.9	82.6	96.4	83.1	60.3	56.8	56.8	51.9	7
8	61.5	67.6	60.3	59.7	67.1	65.7	58.0		58.5	59.0	58.7	59.2	93.6	56.7	56.7	57.9	8
9	52.2	60.4	19.5	19.9	58.1	57.6	19.5	60.7		98.0	82.4	97.2	58.2	59.6	59.6	53.5	9
10	51.2	60.4	19.8	20.2	58.2	57.7	19.8	59.5	2.1		82.1	98.0	58.5	59.6	59.6	52.9	10
11	54.4	60.3	1.8	2.1	61.0	61.6	3.7	60.3	20.2	20.5		82.9	59.2	57.5	57.5	53.2	11
12	52.7	61.0	18.5	18.8	57.7	57.1	19.2	58.9	2.9	2.1	19.5		59.2	59.8	59.8	53.5	12
13	62.8	65.7	60.3	59.7	70.4	68.2	56.8	6.7	61.3	60.7	59.1	59.0		55.6	55.6	59.0	13
14	67.3	41.0	62.7	62.1	4.5	3.4	64.6	65.0	58.2	58.2	62.8	57.7	67.5		100.0	66.5	14
15	67.3	41.0	62.7	62.1	4.5	3.4	64.6	65.0	58.2	58.2	62.8	57.7	67.5	0.0		66.5	15
16	57.4	39.8	73.8	75.3	47.8	46.3	77.1	63.0	73.2	74.9	73.8	73.3	60.1	44.8	44.8		16
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	

1 CH-TW.SEQ
2 D-229E-NC_002645-390bp.SEQ
3 D-BCoV-AF124985-390bp.SEQ
4 D-BCoV-NC_003045-390bp.SEQ
5 D-CCoV-AF124986-390bp.SEQ
6 D-FIPV-AF124987-390bp.SEQ
7 D-HCoV-OC43-AF124989-390bp.SEQ
8 D-IBV-Avian-NC_001451-390bp.SEQ
9 D-MHV-M55148-390bp.SEQ
10 D-MHV-NC_001846-390bp.SEQ
11 D-PHEV-AF124988-390bp.SEQ
12 D-RAT-SDAV-AF124990-390bp.SEQ
13 D-TCoV-AF124991-390bp.SEQ
14 D-TGEV(P)-AF124992-390bp.SEQ
15 D-TGEV-Z34093-390bp.SEQ
16 D-PEDV.SEQ

Table 3

Percent Similarity

Percent Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
1		56.9	72.3	72.3	56.2	56.2	72.3	63.1	68.5	69.2	73.1	69.2	63.1	55.4	55.4	60.8	1
2	63.1		52.3	52.3	73.1	73.1	51.5	53.8	51.5	51.5	53.1	51.5	53.8	72.3	72.3	73.8	2
3	34.6	73.9		100.0	53.1	53.1	96.2	62.3	88.5	89.2	99.2	89.2	61.5	52.3	52.3	57.7	3
4	34.6	73.9	0.0		53.1	53.1	96.2	62.3	88.5	89.2	99.2	89.2	61.5	52.3	52.3	57.7	4
5	64.8	33.4	72.0	72.0		100.0	51.5	53.1	52.3	52.3	52.3	52.3	53.1	99.2	99.2	76.2	5
6	64.8	33.4	72.0	72.0	0.0		51.5	53.1	52.3	52.3	52.3	52.3	53.1	99.2	99.2	76.2	6
7	34.6	75.8	4.0	4.0	75.8	75.8		62.3	86.9	87.7	95.4	87.7	61.5	51.5	51.5	57.7	7
8	50.5	70.1	52.0	52.0	72.0	72.0	52.0		61.5	62.3	62.3	62.3	97.7	52.3	52.3	54.6	8
9	40.8	75.8	12.6	12.6	73.9	73.9	14.4	53.5		99.2	88.5	99.2	60.8	52.3	52.3	56.2	9
10	39.5	75.8	11.7	11.7	73.9	73.9	13.5	52.0	0.8		89.2	100.0	61.5	52.3	52.3	56.2	10
11	33.4	72.0	0.8	0.8	73.9	73.9	4.8	52.0	12.6	11.7		89.2	61.5	51.5	51.5	56.9	11
12	39.5	75.8	11.7	11.7	73.9	73.9	13.5	52.0	0.8	0.0	11.7		61.5	52.3	52.3	56.2	12
13	50.5	70.1	53.5	53.5	72.0	72.0	53.5	2.3	55.0	53.5	53.5	53.5		52.3	52.3	53.8	13
14	66.5	34.6	73.9	73.9	0.8	0.8	75.8	73.9	73.9	73.9	75.8	73.9	73.9		100.0	76.2	14
15	66.5	34.6	73.9	73.9	0.8	0.8	75.8	73.9	73.9	73.9	75.8	73.9	73.9	0.0		76.2	15
16	55.0	32.2	61.4	61.4	28.7	28.7	61.4	68.3	64.8	64.8	63.1	64.8	70.1	28.7	28.7		16
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	

1 CH-TW.PRO
2 229E-NC002645.PRO
3 BCoV-AF124985.PRO
4 BCoV-NC_003045.PRO
5 CCoV-AF124986.PRO
6 FIPV-AF124987.PRO
7 HCoV-OC43-AF124989.PRO
8 IBV-avian-NC_001451.PRO
9 MHV-M55148.PRO
10 MHV-NC_001846.PRO
11 PHEV-AF124988.PRO
12 RAT-SDAV-AF124990.PRO
13 TCoV-AF124991.PRO
14 TGEV(P)-AF124992.PRO
15 TGEV-Z34093.PRO
16 D-PEDV.PRO

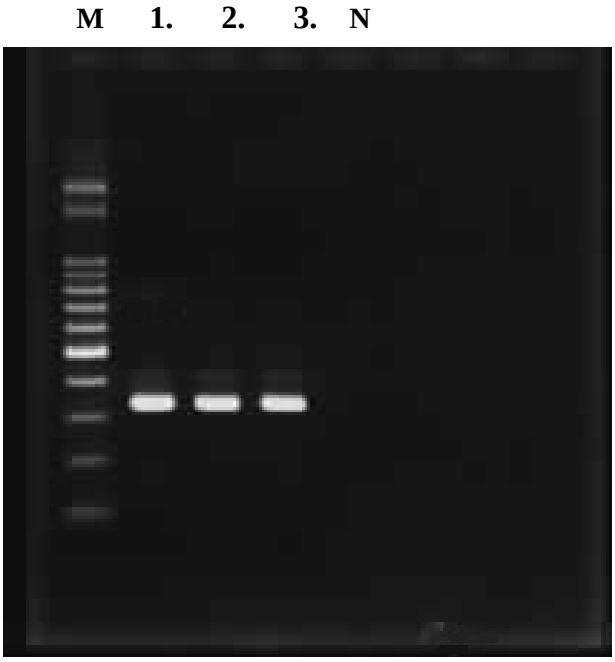

Figure 1. Using F2/R1 as primers to augment by nest-PCR about 368bp section. M is 100bp ladder marker; 1-3 is sputum and two throat swab extracts for a SARS patient. Lane 1 is sputum specimen; Lanes 2 and 3 are first and second throat swabs; N is negative control.

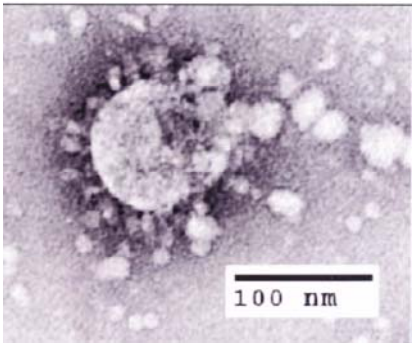

Figure 3. Coronavirus-like Particles Observed in SARS Patients under Electronic Microscopes (source US CDC)

Figure 2. Using Molecular Evolutionary Genetics Analysis (MEGA) version 2.1 for phylogenetic tree analysis. The “neighbor-joining” method was used and bootstrapped 1,000 times.