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Short Communication

Morphology and Morphogenesis of Severe Acute Respiratory Syndrome (SARS)-associated Virus

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Abstract After infecting the Vero E6 cells by nasal/throat swabs collected from SARS patients, we studied the SARS-associated virus by electron microscopy and molecular biological technique. The results show that the diameter of newly isolated virus is about 50nm without envelope or 100 nm with envelope. The virus was proved to be a new coronavirus by RT-PCR and it responded positively to convalescent-phase serum specimen from SARS patients, which is the evidence that this new virus is etiologically linked to the outbreak of SARS. The morphogenesis and distribution of the virus are also discussed in this article.

Key words severe acute respiratory syndrome (SARS); coronavirus; electron microscopy

An acute respiratory infectious disease was first reported in Guangdong province of China in late 2002. The disease is characterized by fever, chest pain, a non-productive cough and dyspnea. A chest radiograph reveals air-space disease. Some patients died from progressive respiratory failure. The disease was first designated “atypical pneumonia (AP)” because of its clinical characterization, and antibiotic treatments are noneffective till now[1,2]. The World Health Organization (WHO) named it as “Severe Acute Respiratory Syndrome (SARS)”. The death rate of this disease is about 3% to 10%, and it spreads rapidly. Over 30 countries had reported SARS cases to the WHO by late April 2003, and there are more than 5000 SARS cases and 300 SARS-related deaths throughout the world. The WHO announced that a new coronavirus mutant is the pathogen of SARS on April 16, 2003[3－7].

We have participated in isolating and identifying the pathogen since February 2003. SARS-related virus was isolated from SARS patients' nasal/throat swabs, and the pathogen of SARS was indicated to be a kind of coronavirus by indirect immunofluorescence and RT-PCR

assay. The results of our study on morphology and morphogenesis of the virus are shown in this article.

1 Materials and Methods

1.1 Material

The nasal/throat swabs were collected from SARS patients in Guangzhou; Vero E6 cells were conserved in the Center for Disease Control of Guangdong Province.

1.2 Methods

1.2.1 *Cell Culture* The nasal/throat swabs were treated with 1000 u/mL penicillin and 1000 mg/L streptomycin at 4 ℃ overnight. After centrifugation at 3000 r/min for 30 min, the supernatant was used to inoculate the monolayer cells at 37 ℃ for 1 h. Remove the solution, and then add 2% bovine serum (Gibco/BRL) into the MEM culture medium (Gibco/BRL). Keep them in CO2 incubator in 33 ℃, and inspect the cells everyday.

1.2.2 *Electron Microscopy* (1) Negative StainingThe culture medium of cytopathic cells was centrifugated at 10 000 r/min for 5 min. About 5 μL supernatant was dripped on a copper grid with Formvar membrane. Negatively stain the sample with 3% PTA. Remove the drop after a few seconds, put the copper grid in a sealed iron box, and then heat it up to 90 ℃ for 1 hour. (2) Ultrathin SectioningThe cytopathic cells were collected by centrifugation at 4000 r/min for 5 min, and fixed with 3.0% glutaraldehyde and 1% OsO4. Dehydrate the samples with ethanol and then embed them in Spurr resin (Sigma Co.). After ultrathin sectioning, the samples were observed under transmission electron microscope. Healthy Vero E6 cells were also treated with as controls.

1.2.3 *Immunofluorescence and PCR Assay* (1) Immunofluorescence Put the cytopathic cells onto a glass slide and then fixed them with acetone at －20 ℃ for 30 min. Dilute the serum samples from SARS patients with PBS to 1∶40, put them in 56 ℃ for 30 min, and then drip them onto the glass slide. Incubate the samples in 37 ℃ for 1 h, and then wash them with PBS for three times. The goat anti human IgG-FITC was added into the samples and incubated at 37 ℃ for 30 min. (2) PCR assayExtract the viral RNA according to the Viral RNA kit made by QIAGEN company, and then use two pairs of coronavirus-specific primers (Roche Co.) to carry out RT-PCR and nest-PCR. PCR products are observed through 1.5% agarose gel electrophoresis. The primers of RT-PCR and nest PCR are listed below respectively:

BNIoutS/BnoutAs:

sense, ATGAAT TAC CAA GTC AAT GGT TAC

antisense, CAT AAC CAG TCG GTA CAG CTA C

BNIinS/BNIAs:

sense, GAA GCT ATT CGT CAC GTT CG

antisense, CTG TAG AAA ATC CTA GCT GGA G

2 Results

2.1 Results of Negative Staining

In the supernatant of cytopathic Vero E6 cells, virus particles can be seen through transmission electron microscope (TEM). The diameter of the virus is between 60 nm to 120 nm. The virus particles can be of round or oval shape. There are many 10－20 nm cilia-like

protrusions outside the envelope. The protrusions are arranged in order and there are wide gaps between them (Fig.1). This appearance is extremely resembled to coronavirus.

2.2 Results of ultrathin sectioning

The ultrathin sections of Vero E6 cells are observed through TEM. The cytopathic cells have many empty vesicles. Mitochondria are fewer than normal cells, and the outer membrane and crista of mitochondria are disrupted. Endoplasmic reticula decrease and always swell. Other organelles are hard to be seen. There are fingerprint-like fibres in empty vesicles and cytoplasm. The chromatins in nucleus shrink and congregate along the edge, and sometimes some tiny granules appear in the nucleus. However, in normal cells, the empty vesicles are fewer, mitochondria are intact, and no pathological change or virions are found.

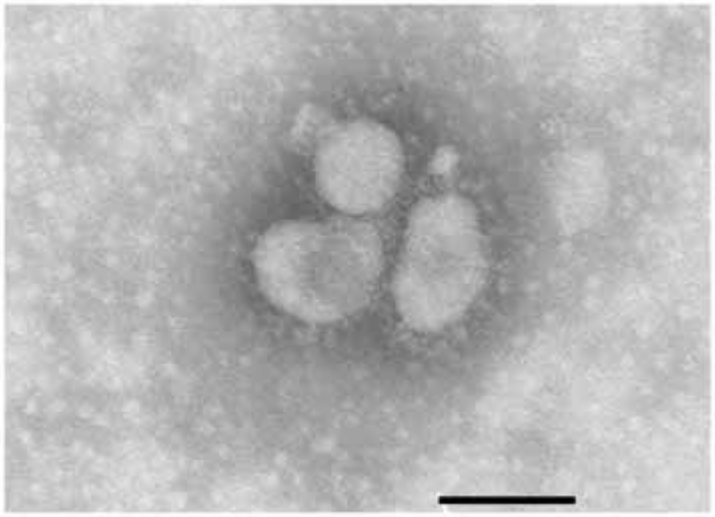

Fig.1 Negative staining of SARS-associated virus（Bar=100 nm）

Many virus particles are found both inside and outside the cytopathic cells. The virions located outside the cells always line up along the cell membranes, most of them have envelope, and their diameter is between 60 nm to 100 nm, whereas the length of the protrusions in envelope is between 10 nm to 20 nm. These characteristics are consistent with those of the negatively stained viruses. Nucleocapsids are overcolored, always round in shape, and the diameter is between 45 nm to 70 nm (Fig.2).

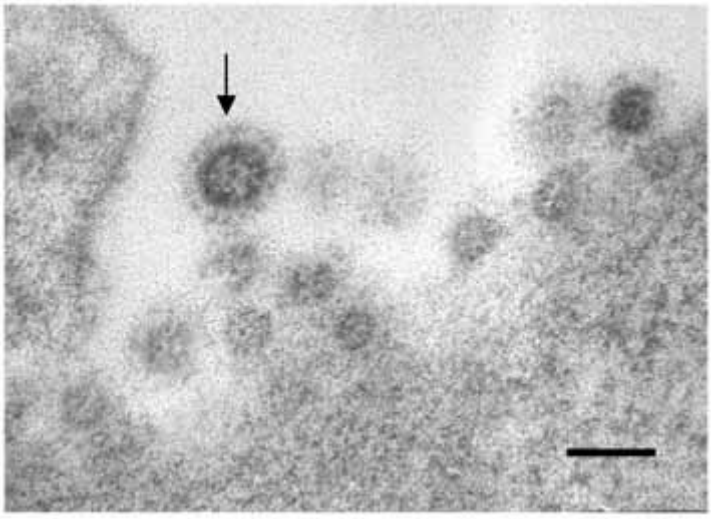

Fig.2 Virions outside the cell, and the arrow points to a typical virion with envelope (Bar=100 nm)

Virus particles always congregate, and form a virus vesica with an encircling membrane. In the vesica, virus particles do not have envelope and the diameter of them is about 50 nm. Staining of the virus differs: some are light-colored in the center as if they are hollow; some are overcolored with petal-like pattern (Fig.3). Some vesica membranes are clear, and the viruses inside are also clear and always light-colored (Fig.4); whereas, some vesica membranes are blurry, and the virus inside are misty and overcolored. There are some empty vesicles near these vesicae. Most of the virions inside these empty vesicles have envelope, and their appearance is consistent with that of the virus outside the cells (Fig.5,6). These empty vesicles are similar to the “smooth vesicles” reported[8]. Therefore, we suppose the vesicae are related to the virus morphogenesis, and name them as “virus morphogenesis matrix vesicae (VMMV)”.

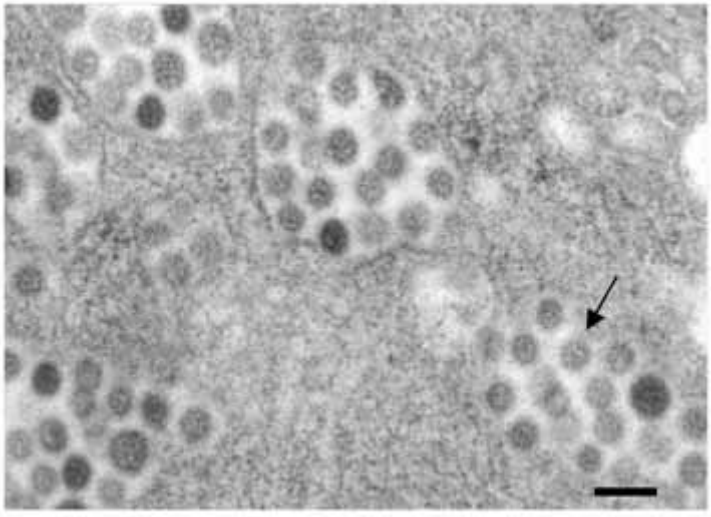

Fig.3 Virions in the VMMVs, and the arrow points to a virus particle with petal-like pattern（Bar=100 nm）

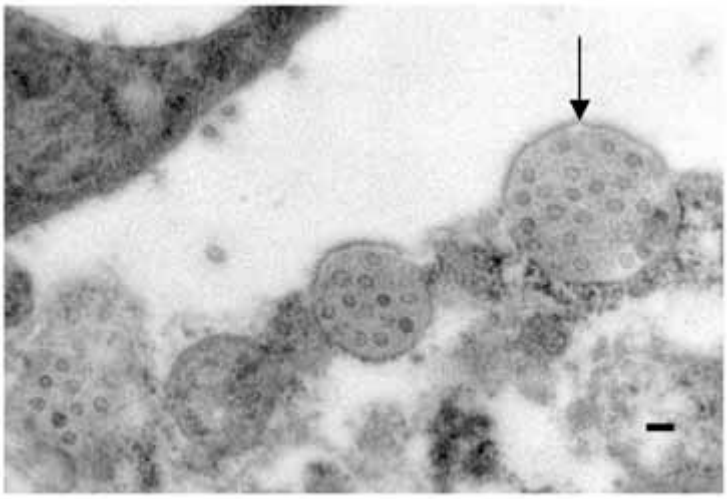

Fig.4 VMMVs with clear membrane（Bar=100 nm）

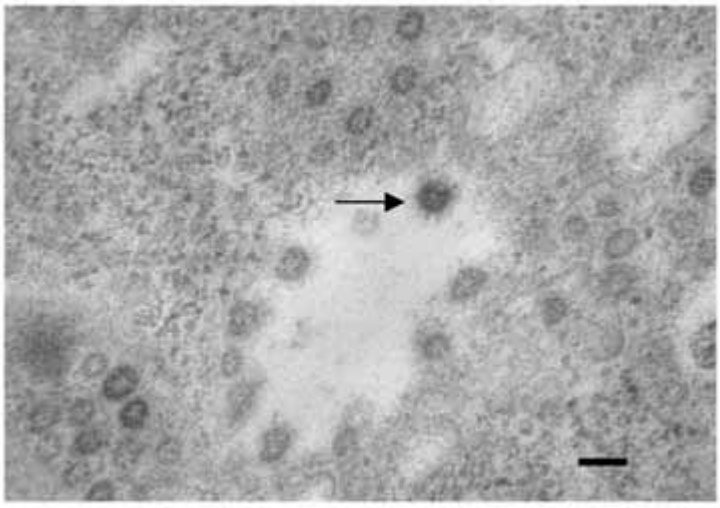

Fig.5 Smooth vesicle and the virions inside, and the arrow points to a virus particle with envelope（Bar=100 nm）

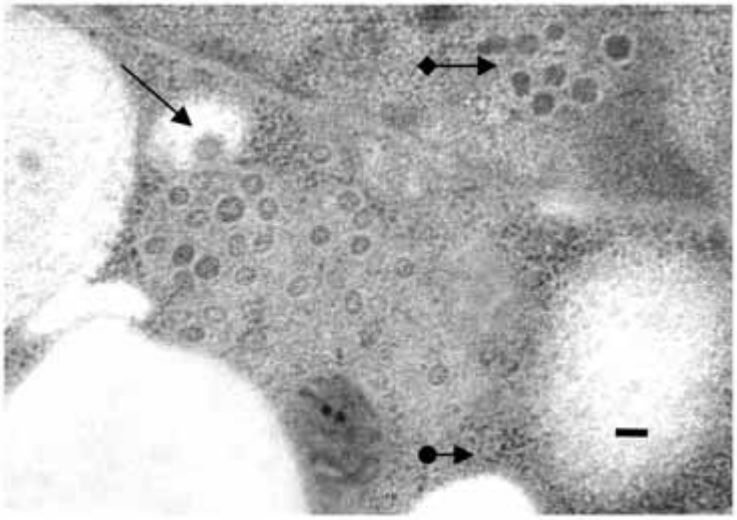

Fig.6 Smooth vesicles and the enveloped virion inside （→） near the VMMVs, and there are virus particles in the nucleus （ ●→ ） （Bar＝100 nm）

Virus particles were also found in cytopathic nucleus, these viruses are overcolored, and there are light-colored distinct gaps between them and the chromatins (Fig.7). Furthermore, one special phenomenon is that the nucleic membranes swell to form bubbles, some bubbles are very long and most of the bubbles contain SARS-associated virus. These bubbles differ in size, and they contain several to tens of virus particles, most of these virions are light-colored (Fig.8).

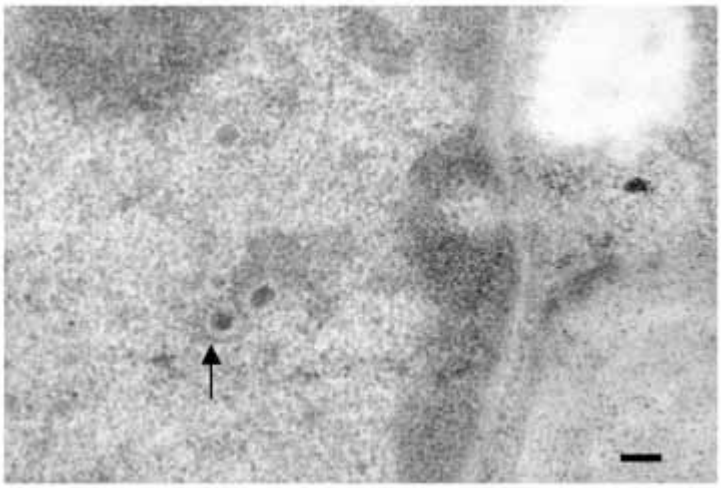

Fig.7 Virus particles in a nucleus （→）（Bar=100 nm）

No other suspect pathogen particles are found except for the virus mentioned above in cytopathic Vero E6 cells. However, there are no such virions or other suspect pathogen particles in normal Vero E6 cells.

2.3 Indirect Immunofluorescence and RT-PCR Assay

The results of indirect immunofluorescence assay indicate that convalescent-phase serum collected from 10 SARS patients respond with the infected cells positively. But the results of 20 healthy individuals' serum are all negative.

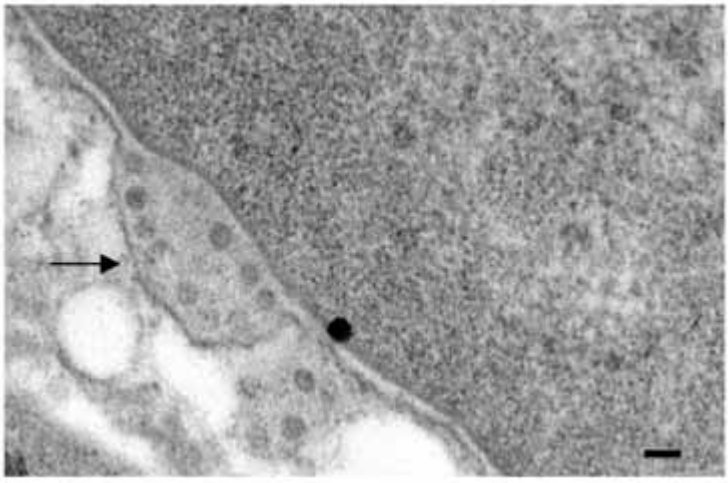

Fig.8 Nucleic membrane swells to form a bubble(→) with virions inside (Bar=100 nm)

The 1.5% agarose gel electrophoresis of RT-PCR product shows that one 110 bp fragment can be seen with EB staining, which is consistent with the 110 bp target of BNIinS/BNIinAs.

3 Discussions

(1) All the cytopathic cells contain the same kind of virions, and no other pathogens are found in these cells. Furthermore, normal cells do not contain this kind of virus particles. Therefore, these virus particles may probably be the SARS pathogen, and their appearance is similar to the coronavirus reported in the literature [9,10].

(2) The primers of RT-PCR and nest-PCR are designed according to the Replicase 1B conservative sequence of coronavirus, and the target fragment is 110bp. A specific 110bp fragment is obtained as the product of RT-PCR and nest-PCR, which means the virus is coronavirus. According to the genome sequencing and sequence analysis (published in another article), this virus is a new coronavirus.

(3) Using the virus in cytopathic cells as antigen, with serology method, we test the IgG antibody specimen from SARS patients' convalescent-phase serum and healthy individuals' serum. The positive percentage of patients' serum antibody against this virus is 100%, whereas no health individual's serum contains this antibody. This result is similar to those of other laboratories in the world. Combined with the results of overseas labs[3,6,11–14], the isolated coronavirus is probably the main pathogen of SARS.

(4) However, it is still being argued that the new SARS-associated virus is the only pathogen of SARS[15,16].In our study, we also found other virus-like particles and pathogens in pathologically changed tissues of SARS patients and other cultured cells, such as Hep-2, MDCK (data not shown). Other pathogens are not found in Vero E6 cells maybe because the cell strain is only sensitive to SARS-associated coronavirus, not sensitive to any other pathogen.

(5) On the basis of the whole research, we suppose the virus morphogenesis process as below: at first the premature virus particles appear in the VMMVs, and these particles are relatively small, light-colored in the centre and hollow. As the virus grow in the VMMVs, nucleic acid and protein assemble gradually; therefore, the virus become bigger and overcolored. After maturation, the viruses obtain envelope and locate in the smooth vesicles. At last, virus particles release from the cells. We are studying which functional proteins play an important role in virus morphogenesis. This study is of great importance in drug design against SARS-associated virus.

(6) It has not been reported previously that SARS-associated virus or any RNA virus appear in swelled nucleic membrane bubble or nucleus. It is thought that coronavirus nucleic acid duplicate in cytoplasm, and the whole assembly process also takes place in cytoplasm[8,17]. Consequently, coronavirus particles should not appear in nucleus. However, virus particles are found in some nuclei and nucleic membrane bubbles in our research. One possible explanation is that the SARS-associated virus is a new coronavirus, and its duplication, assembly site and morphogenesis have changed.

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