

Reovirus, isolated from SARS patients

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Abstract Beijing has been severely affected by SARS, and SARS-associated coronavirus has been confirmed as its cause. However, clinical and experimental evidence implicates the possibility of co-infection. In this report, reovirus was isolated from throat swabs of SARS patients, including the first case in Beijing and her mother. Identification with the electron microscopy revealed the characteristic features of reovirus. 24 of 38 samples from other SARS cases were found to have serologic responses to the reovirus. Primers designed for reovirus have amplified several fragments of DNA, one of which was sequenced (S2 gene fragment), which indicates it as a unique reovirus (orthoreovirus). Preliminary animal experiment showed that inoculation of the reovirus in mice caused death with atypical pneumonia. Nevertheless, the association of reovirus with SARS outbreak requires to be further investigated.

Keywords: reovirus, coronavirus, SARS, electron microscopy.

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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, Hong Kong, and Beijing, of severe febrile respiratory illness that spread to household members and health care workers. The syndrome was designated “severe acute respiratory syndrome” (SARS) by WHO in March 2003, and global efforts to understand the cause of this illness and prevent its spread were instituted in March 2003^[2]. Since then, an international team of researchers has completed the final proofs that SARS is caused by the primary suspect—SARS-associated coronavirus (SCV), a novel coronavirus^[1–3].

Many cases in Beijing can be linked through chains of transmission to a jewelry-business girl, who once visited Guangdong Province and then came to Beijing, where

she was hospitalized with SARS as the first case in Beijing. Though she survived and recovered from SARS, her father and mother died of SARS, and more than dozens of her relatives and health care workers were infected with SARS by her. SCV was isolated in lung tissue collected at autopsy from her father, and the first isolate in Beijing was named the BJ01 strain of SARS-associated coronavirus, whose genome sequence has been determined^[4].

Although SCV has been named publicly by WHO and member laboratories as “SARS virus” (press release issued by WHO April 16, 2003)^[5], about 60% of SARS cases in Beijing, diagnosed by the symptomatic case definition of the disease, cannot be traced back to a known SARS case^[1]. Moreover, more than 19.6% patients with SARS had diarrhea^[6,2], but in most cases, SCV cannot be isolated from the stool of those patients^[3]. The question is whether some of the cases are caused by a different kind of virus, or co-infected by other virus (speaking by David Heymann, executive director of Communicable Diseases for WHO, in May 19, in Geneva)^[1]. In this report, we describe our efforts in the isolation of reovirus from SARS patients in Beijing.

1 Materials and methods

(i) Materials. Throat swabs used in the experiments were taken from the first SARS case in Beijing and her mother, when they had been hospitalized for 2 d due to a fever. Hep-2 and Vero-E6 cell lines were from Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences, China. Serum samples of SARS patients were from Beijing Youan Hospital, 302 and 309 hospitals.

(ii) Methods. Isolation of reovirus: To isolate viruses associated with SARS, we inoculated the clinical specimen (material from throat swabs) obtained from the first patient with SARS admitted to hospital in Beijing onto Hep-2 cells. All cultures were observed daily for cytopathic effect. Any cultures exhibiting identifiable cytopathic effect were subjected to several procedures to identify the cause of the effect.

RT-PCR: PCR reactions. The detection of conserved genome sequence (S2) was carried out using the reaction mixture (50 μ L in total volume) as follows: 5 μ L 10 \times PCR reaction buffer, 1 μ L 10 mmol/L dNTP, 2 μ L 25 mmol/L MgCl₂, 1 μ L Taq enzyme (2.5 U), upstream primer 1 μ L, downstream primer 1 μ L, 2 μ L cDNA. The PCR reaction conditions were as follows: hot start (95 $^{\circ}$ C 5 min), denaturing at 95 $^{\circ}$ C for 30 s, annealing at 56 $^{\circ}$ C for 30 s and extending at 72 $^{\circ}$ C for 30 s. The cycle was re-

1) Walgate, R., Many Beijing SARS case can't be traced back, The scientist, Daily News May 20 2003. <http://www.biomedcentral.com/news/20030520/03>

2) Hong Kong SAR Government Information Center, Main Findings of an Investigation into the Outbreak of Severe Acute Respiratory Syndrome at Amoy Gardens. http://www.info.gov.hk/info/ap/pdf/amoy_e.pdf (access May 28, 2003).

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peated for 30 times and finally the reaction was incubated at 72°C for 7 min. Sequences were determined by Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences, China.

Electron microscope: Samples were processed by standard techniques including fixation, dehydration, embedding and ultrasectioning. The sections were observed under Philips electron microscopy. The control samples were observed together.

Animal experiments: Four-week female Balb/C mice were maintained under specific-pathogen-free conditions.

Animals were divided into three groups, 5 mice in each group. They were inoculated 0.5 mL of reovirus by an intraperitoneal injection.

2 Results and discussions

We isolated the virus in throat swab specimen from the first case in Beijing and her mother, and initially considered it as a possible variant of SCV, but the PCR test failed to amplify DNA from it. After the subsequent demonstration of a serologic response to this virus in other SARS patients, which suggested a possible association

Fig. 1. Detection of reovirus by electron microscope. (a) Electron micrograph of the Hep-2 cells infected with the reovirus isolated from the throat swab specimen of the first case in Beijing shows aggregate of viral particles with a few microtubular structures (arrows) in the cytoplasm close to the nucleus (N). Most of the reovirus with electron lucent core was in the process of assembling, and the others were matured viral particles with dense central core. (b) Showing numerous matured reovirus accumulated in the cytoplasm of the cells above. (c)—(d) Showing reovirus particles aggregated in a crystalline-like array. (e) Showing the matured reovirus particles, 60—80 nm in diameter, with a dense central core and a thick outer coat, capsid. (f) Showing coexistence of reovirus (Re) and coronavirus (Co) in the cytoplasm of the cells infected with an individual strain of SCV sample. Inset: Reovirus (right upper), coronavirus (left lower).

between this virus and SARS, we made a further investigation on it. Electron microscopy (EM) examination of Hep-2 cells and Vero-E6 cells infected with the virus isolated from throat swab specimens of the first case in Beijing revealed characteristic reovirus particles with a size of about 60–80 nm in diameter. Fig. 1(a)–(c) shows aggregates of reovirus particles in the cytoplasm of Hep-2 cells. The reovirus is observed in different mature stages, and two types of viral particles can be seen: the matured particles with dense central core, and the incompletely assembled virus particles with lucent core, both of which have capsid about 14 nm in thickness. Most of the viral particles were found in large clusters, which arranged in crystal-arrayed form (Fig. 1(c)–(e)). The reovirus was also isolated by the same way from her mother, but SCV could not be isolated from throat swab specimens of both of them. In Vero-E6 cells infected with strain of SCV, typical coronavirus particles of 80–120 nm in diameter with a dark central nucleocapsid and envelope were seen (Fig. 1(f)). It is important to note that coronaviruses within a vacuole and reovirus particles in cytoplasm were observed simultaneously in Vero-E6 cells infected with an individual SCV sample (Fig. 1(f)), while there was neither of these viruses in control Vero-E6 cells.

The serologic response to the reovirus was tested by a standard ELISA technique with serum samples from 38 other patients with SARS and 35 randomly selected healthy controls. The average A value of control samples is 0.047. The patients with SARS were all diagnosed by the symptomatic case definition of the disease. After screening of the 38 serum samples from SARS patients for serologic response, we found that 24 samples were positive to the reovirus (>2.1 times of the control A value, 63%) (Fig. 2). Only one sample from the control healthy samples was positive to the reovirus.

To further confirm reovirus infections in the first case in Beijing and her mother, primers were designed to

amplify conserved genome fragments of reovirus. A fragment of about 488 bp was obtained by RT-PCR with the primers: (1) 5'-GTT GGA TTT GGT GGT CTG C-3'; (2) 5'-CCA CTC CAC ATA TCC TCG-3', which is consistent with the theoretical value of S2 gene. Moreover, several other segments of the reovirus genome were amplified by RT-PCR. We got the following sequence by DNA sequencing: 5'-aaattggaccgaaccccgtaagttagtaacgttacggcactcaattgctggtacactcgggtcctaaatcttgatcacgtgctaagaatggtaatcgtcgcaatcatacactcgtatcgttgcgcctgcaaacgaggctgtgccagaa-ccttggtggggctacaactaaaccatcaagcgtaggagctgaccacactaggc-gcaggaaacctgatctccccatcggtggttagccgaaatggtatctgaagag-taccacttaaaaggcaactcattcgataatcttgaacctcggtcggacagag-agccgatg-3' (GenBank accession number: AY335545). The Blast search shows that this sequence is of 87% homology with orthoreovirus S2 gene fragment in database, indicating it as a unique reovirus.

Preliminary animal experiment was also carried out with the reovirus. 10 Balb/C mice died of with atypical pneumonia within 21 days post inoculation of the reovirus. Under the microscopy, the alveolar septa were thickened, and large amounts of mononuclear cells and comparatively less polymorphic nuclear cells were observed in the alveoli. Fibrosis could be observed under EM. The alveoli structure of mice in control group was intact. More animal experiments need to be further performed, especially the relationship between virus dose, inoculation way, infection time and lung pathological changes awaits exploring.

The isolation of reovirus from the throat swab specimens of patients with SARS and the subsequent demonstration of this virus and serologic response in other SARS patients to this virus showed a possible association between this virus and SARS. It is known now that early in 1967, a case of fatal interstitial pneumonia caused by reovirus infection was reported^[7]. In recent years, animal models of lung fibrosis, ARDS and BOOP were set up^[8,9]. Lamirande et al. isolated a strain of pathogenic reovirus

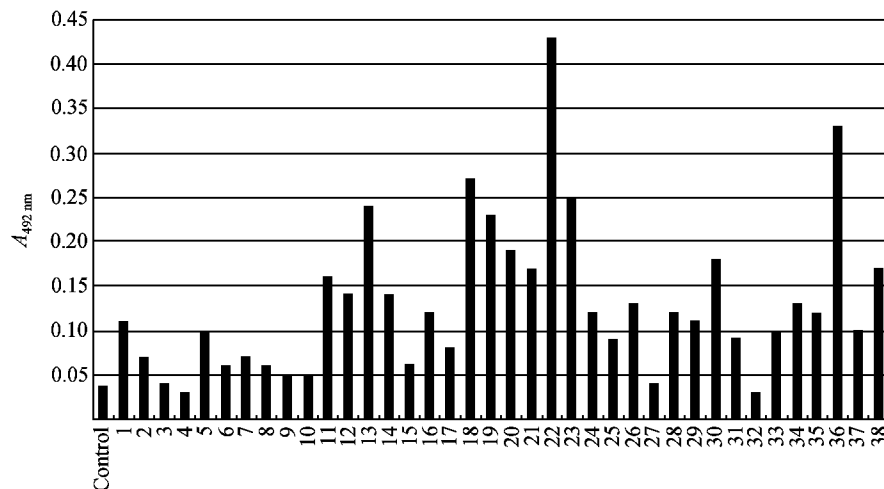


Fig. 2. ELISA detection of serologic response to the reovirus in 38 patients with SARS.

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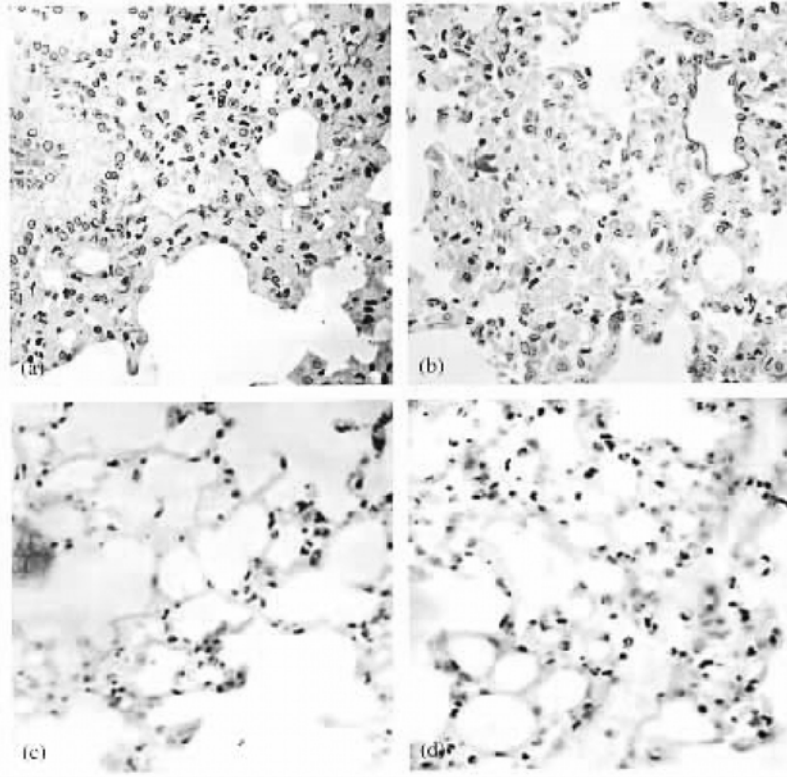


Fig. 3. Observation of lung tissue from Balb/C mice infected with the reovirus ((a) and (b)) for 21 days and from control mice ((c) and (d)) (magnification: (a) and (b) $\times 100$; (c) and (d) $\times 200$).

from ratsnake^[10]. A juvenile black ratsnake (*Elaphe obsoleta*) was experimentally inoculated with the isolate and was found dead 26 days post inoculation. Necropsy revealed diffuse sub-acute interstitial pneumonia. It was the first report on experimental transmission of reptile reovirus. The most characteristic and deadly pathological changes are ARDS and acute lung fibrosis. Therefore, the reovirus isolated from patients with SARS needs to be further investigated, which may bring some insight into the mechanisms of SARS outbreak.

3 Perspectives

The finding of the study provides evidences of an involvement of reovirus co-infection in SARS, however, large studies with strict control groups are needed to verify the correlation of reovirus with SCV, and their roles in SARS outbreak. Meanwhile, we should bear in mind that there still have some doubts on the role of the reovirus in SARS outbreak. Thus, much work requires to be done in order to clarify the correlation of the reovirus infection with SARS outbreak.

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