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Preparation and characterization of SARS in-house reference antiserum

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1. Introduction

SARS is a severe acute respiratory infective disease. A safe and effective vaccine would be an important complement to the existing SARS control strategies [1]. Neutralization antibodies against SARS virus in immunized animals are measured by potential serological assays. A well-characterized SARS reference antiserum is of great necessity to provide reliable standardized evaluation and quality control for the assay, and moreover to instruct the development of inactivated vaccine against the SARS virus.

The current report summarizes preparation, characterization and calibration of a SARS reference antiserum, according to the WHO biological reference requirements [2].

2. Materials and methods

2.1. Viral strains and virus manipulation

Viral strains: SARS viral strains Sino1, Sino2, Sino3 and Sino6 were isolated from pharyngeal swabs of clinically confirmed SARS patients at Peking Union Hospital. SARS virus was propagated on Vero cells in a culture flask cultivated at 37 °C until 75% cells showed cytopathic effect. After three freeze–thaw cycles, virus was harvested and well characterized. The batch of virus was stored at −80 °C as reference virus (manuscript in preparation) for neutralization assay.

After harvest, the SARS virus was inactivated and the total protein was purified by centrifugation, ultrafiltration and gel filtration. The gel filtration eluent was used for Western blot assay.
2.2. Cell line

Vero cells, a line of African green monkey kidney cells, were obtained from ATCC (American Type Culture Collection). Vero cells were grown at 37°C in minimum essential medium (MEM), containing 10% (w/v) fetal calf serum and 1% (w/v) glutamine.

2.3. Serum

A panel of positive sera, collected from 20 patients in the convalescent stage of SARS, was heat inactivated at 56°C for 30 min and stored at −20°C. In order to verify the inactivation efficiency, Vero cells were inoculated with the inactivated serum and cultivated for three generations. No cytopathic effect was detected. In addition, a panel of serum samples was collected from 10 healthy people for negative control.

2.4. Titration of infective virus

Vero cells were cultivated in 96-well plates at 37°C to form confluent monolayer. Cells were inoculated with 200 μl per well of viral suspension that was reconstituted and diluted serially in tenfold steps in MEM containing 2% new-born calf serum and 50 μg/ml penicillin (pH 7.2), with each dilution filling 8-well. The 96-well plates were incubated at 37°C in 5% CO2 incubator. Virus titre was expressed as cell culture infectious dose (CCID), which is the dilution of virus causing half of the cultured cells to produce CPE (cytopathic effect).

2.5. Neutralization assay [3,4]

Heat inactivated sera (two-fold serial dilution from 1:2 to 1:2048, 100 μl for each dilution) were added to equal volume of virus (diluted to 100CCID50/μl) and incubated for 1h at 37°C before being added to confluent monolayers of Vero cells in 96-well plates and incubated at 37°C in 5% CO2 incubator for 1–4 days. During the incubation period the cell cultures were checked for development of CPE (cytopathic effect).

2.6. Western blot assay

Proteins from SDS-PAGE gels were transferred onto nitrocellulose membrane. For immunostaining of the transferred proteins, the blocked membrane was incubated with convalescent serum. Anti-human antibody conjugated with HRP (horseradish peroxidase, Sigma) was used as the second antibody, and the color developing substrate was DAB.

2.7. ELISA

The convalescent serum of SARS was identified and confirmed by a commercially obtained enzyme-linked immunosorbent assay (ELISA) Kit (Beijing WanTai). The antigenic protein employed in the ELISA kit was part of the recombinant N protein of the SARS CoV (the polypeptide product of the third ORF of the N protein coding region) expressed in an Escherichia coli strain. The purified SARS virus protein was immobilized on a 96-well plate. The antiserum against SARS virus was added to the coated wells and incubated. HRP-labelled SARS antigen was then added to react with the bound antiserum. TMB was used for color development.

2.8. Preparation of reference antiserum

The serum STS-D-Zhang-05 was mixed thoroughly and filled into ampoules (0.5 ml/ampoule). The serum was freeze-dried and the ampoules were sealed.

2.9. Moisture content

The moisture content of freeze-dried reference antiserum was detected by Karl Fischer method described as previously [5]. The moisture content of at least three ampoules was tested separately.

2.10. Stability test

The stability of the reference antiserum was tested by an accelerated degradation test using temperature at −20, 37 and 56°C, for 7, 14, 21 and 28 days of storage, respectively. Potency was determined by neutralization assay. All samples were monitored against the −20°C stored samples.

3. Results

3.1. Identification of SARS corona virus

The viruses were electron microscopically visualized, and appeared to have clearly Corona virus typical characteristics. Cytopathic changes could be seen when the viruses were inoculated on Vero cells, which are susceptible to SARS virus infection. The two virus strains, Sino1 and Sino3, which we used extensively for our research have been sequenced (they have more than 99% similarity with the available SARS CoV virus sequences) and have been accepted by GenBank with the accession number of AY485277 (Sino1) and AY485278 (Sino3). The virus could neutralize convalescent sera. All the above evidence has proved that the virus we used is SARS corona virus.

3.2. Epidemiological identification

The antiserum was from a male SARS patient with clear epidemiological history and was confirmed clinically. This male patient was identified as a SARS probable case according to the WHO criteria and was confirmed to be SARS case according to the diagnosis criteria of the Chinese Ministry of Health. He had been infected SARS through contact with a SARS patient at Zhangjiakou No. 2 Hospital affiliated with
Zhangjiakou Medical Institute. There were two other patients who had had close contact with him. They also showed SARS symptoms such as fever, cough and dyspnea and pulmonary could be detected by X-ray check. They were also confirmed to be SARS probable cases according to the WHO criteria and were confirmed to be SARS cases according to the diagnostic criteria of the Chinese Ministry of Health. All patients were interviewed to ascertain their contacts with each other.

3.3. Serological assays

Convalescent serum was collected from the patient in 3.2, and was named STS-D-Zhang-05 after inactivation process. Antibody specificity of the serum was identified by ELISA, Western blot assay, and neutralization assay.

The convalescent sera were collected from 20 SARS patients (probable cases according the WHO criteria) in the Inner Mongolia Autonomous Region (NeiMengGu, thus the three letter initial code Nei), the cities of Beijing and Zhang Jiakou (thus the three letter initial codes Jing and Zhang, respectively). The collection was arranged by Chinese Ministry of Health and China CDC. Sera were collected 1–5 months post the onset of the symptoms.

Nineteen sera from the above 20 sera tested gave positive results and all negative ones proved negative. Results are given in Table 1. The serum STS-D-Zhang-05 had strong positive reaction, confirming SARS antibody specificity. The serum volume of STS-D-Zhang-05 is sufficient for reference preparation and its neutralization potency is close to the potency GMT (1:54) of all the 19 positive sera.

Western blot assay was further performed to identify the SARS antibody in the serum STS-D-Zhang-05. A band of 48 kDa corresponding to the SARS N protein (Nucleocapsid protein) was detected, suggesting the existence of specific anti-SARS antibody. No bands were detected for the negative control serum. The result is shown in Fig. 1.

Neutralization assay demonstrated that the serum is capable of neutralizing four SARS-CoV strains (Table 2).

3.4. Preparation of SARS reference antiserum

After the above identification, STS-D-Zhang-05 was designated to be SARS reference antiserum candidate. The serum, which had a volume of about 100 ml, was filled into ampoules, freeze-dried, sealed, and tested for residual moisture following the WHO requirements [2]. In total 163 ampoules were obtained. This batch of reference antiserum, assigned a lot number of 20031009, was stored at −20 °C and was in the charge of a special keeper.

3.5. Calibration of SARS reference antiserum

Neutralization assay was used to calibrate the reference antiserum. Repetitive titrations were performed by different persons at three different times. Statistic analysis identified the range and GMT (geometric mean titre) of the antibody potency. Results are given in Tables 3 and 4.

3.6. Potency definition of SARS reference antiserum

The potency of SARS reference antiserum is defined to be 52.7 U. The geometric mean titre is 1:52.7, which is

Table 1
Neutralization test results for 20 convalescent sera from SARS patients

| Serum code | NT potency | Serum code | NT potency | Serum code | NT potency |
|------------|------------|------------|------------|------------|------------|
| STS-D-Nei-01 | 1:25 | STS-D-Nei-02 | 1:32 | STS-D-Nei-03 | 1:128 |
| STS-D-Nei-04 | 1:10 | STS-D-Nei-05 | 1:64 | STS-D-Jing-05 | 1:51 |
| STS-D-Jing-06 | 1:203 | STS-D-Jing-07 | 1:64 | STS-D-Jing-08 | 1:51 |
| STS-D-Jing-09 | 1:64 | STS-D-Jing-10 | 1:51 | STS-D-Jing-11 | 1:51 |

The potency GMT of all the 19 sera is 1:54.

* This is the negative serum.
Table 3

Results from titration by different persons at different times

| Test  | Number of experiments | GMT  |
|-------|-----------------------|------|
| A     | 11                    | 1:52.3 |
| B     | 4                     | 1:50.6 |
| C     | 6                     | 1:54.6 |

Table 4

Results from titration by the same person at the same time (intra-test) or at different times (inter-test)

| Test number | Result          |
|-------------|-----------------|
|             | Intra-test      | Inter-test    |
| 1           | 1:80            | 1:51          |
| 2           | 1:44            | 1:40          |
| 3           | 1:64            | 1:64          |
| 4           |                | 1:64          |
| 5           |                | 1:51          |
| 6           |                | 1:51          |
| 7           |                | 1:32          |
| 8           |                | 1:51          |

Geometric mean: 1:60.8
Coefficient of variance: 0.288

Test number indicates independent measurements.

3.7. Moisture content

The moisture of the freeze-dried SARS reference antiserum was determined to be 0.5%.

3.8. Stability test on SARS reference antiserum

Results on the stability of SARS reference antiserum at 37 and 56 °C are given as Table 5. More experiments on stability are still in progress.

Results in Table 5 show that neutralization antibody potency has not decreased substantially after SARS reference antiserum was kept at 37 and 56 °C for 0–35 days. Therefore, the storage condition for SARS reference antiserum is designated to be −20 °C in the dark, and the time period before expiration is primarily designated to be 1 year.

Table 5

Results on the stability of SARS reference antiserum

| Experimental duration (day) | Neutralization Antibody Potency at different temperatures |
|-----------------------------|----------------------------------------------------------|
| 0                           | −20 °C: 1.65 1.45 1.45 1.45 1.45 1.45 |
| 7                           | −20 °C: 1.65 1.51 1.32 1.51 1.51 1.51 |
| 14                          | −20 °C: 1.65 1.23 1.23 1.16 1.16 1.13 |
| 21                          | −20 °C: 1.65 1.23 1.23 1.16 1.16 1.13 |
| 28                          | −20 °C: 1.65 1.23 1.23 1.16 1.16 1.13 |
| 35                          | −20 °C: 1.65 1.23 1.23 1.16 1.16 1.13 |

4. Discussion

The world has to be prepared to face a possible resurgence of local SARS epidemic. Although progress has been made worldwide to develop a safe and effective human vaccine for SARS prevention, there still lacks international references for and quality control and expressing test results. A recent WHO SARS laboratory workshop has agreed on the urgent need for quality control, standardization of test protocols and reagents, the need for verification of initial cases/clusters in non-epidemic periods, and the establishment of international reference and verification laboratories [6]. Antibody capable of neutralizing the virus has been discovered in the convalescent serum from SARS patients, suggesting its role in the immunity to SARS infection. In order to fulfill the needs for standardization, we have been working on a potential reference antiserum in an effort to search for a vaccine to combat SARS.

A SARS reference antiserum, which will serve as a reference in different serological methods in immunized animals, has been successfully prepared, tested and calibrated.

Qualitative tests including ELISA and Western blot assay and neutralization assay have made it clear that the reference antiserum has satisfied the basic criteria for references. According to the titration results, the neutralization antibody unit of the reference antiserum is 52.7 U. Primary results on stability showed that neutralization antibody potency of the reference antiserum has not decreased substantially up to 3 weeks. The stability test is still in progress.

This newly developed reference antiserum is so far only for in-house use. For acceptance to become an international reference candidate, more has to be done to comply with the WHO regulations on references, such as collaborative titration of the serum and methodology verification.

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