Letter to the Editor

A PCR-based Rapid Neutralization Assay for GII.4 Norovirus Infection in HIEC6 Cell Culture

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Because of limited viral replication and lack of cytopathic effect in cell culture, a new PCR-based rapid seroneutralization assay for detection of GII.4 norovirus neutralized antibodies was developed with serum samples from acute-phase patients, convalescent-phase patients and healthy controls. According to this study, neutralizing antibodies were detected in 100% of convalescent-phase sera, and in 2.5% of healthy controls sera. However, all of the acute-phase serum samples could not neutralize virus efficiently. Compared to the results from ELISA (96.2% at sensitivity and 80% at specificity), the present *in vitro* neutralization assay is more specific and more sensitive.

Norovirus (NoV), a member of the family *Caliciviridae* in the genus *Norovirus*, is now recognized as a leading cause of nonbacterial acute gastroenteritis and affects millions of people worldwide. The virus is classified into five genogroups and then subdivided into more than 30 genotypes. Strains responsible for human gastroenteritis essentially belong to genogroups GI and GII, and genotype GII.4 NoV is the major cause of infections[1-2]. Until now, an *in vitro* NoV seroneutralization assay has been severely hampered because of the lack of a suitable NoV cell culture system. In a previous study, several cell lines such as HIEC6, a glandular epithelial cell line, showed the ability of replicating NoV. However, only up to 2-$\log_{10}$ increase in viral genomic RNA level was detected in culture supernatant and no observable cytopathic effect was noted in this cell line[3]. Herein, we described a new PCR-based rapid seroneutralization assay, which could be used for the detection of GII.4 NoV neutralized antibodies in HIEC6 cell culture.

Ethical approval for this study was given by Suzhou Kowloon Hospital, China. After written informed consents were obtained from the individuls or their legal guardians, 26 hospitalized patients (aged from 3-6 years) infected by genotype GII.4 NoV were selected during a NoV outbreak in Suzhou, China in the summer of 2012. All of the cases were diagnosed and genotyped by Suzhou Center for Disease Prevention and Control and confirmed by Jiangsu Center for Disease Prevention and Control. The acute-phase serum samples were collected from the patients when they visited the hospital (Day 1) and the convalescent-phase serum were collected 15 days later (Day 15). Meanwhile, 40 serum samples from healthy children (aged from 3-5 years) were collected from the same region. All of the samples were stored at -70 °C until analysis. The GII.4 NoV stool suspension (used as control), with 5.0×10^7 copies/mL of virus titer was also stored at -70 °C in our laboratory.

The *in vitro* PCR-based rapid seroneutralization assay is similar to the method for HEV neutralizing antibody detection as described before[4]. Briefly, 100 cell culture infectious doses (1.5×10^5 copies/mL) of a NoV inoculum was determined as described in previous study, and then diluted in 100 μL of PBS were mixed with 100 μL of a serum sample diluted in PBS at 1:10. After incubation at 37 °C for 1 h, the mixture was inoculated onto a cell monolayer of HIEC6 (gift from CDC, USA) cultured in 6 wells plates (ThermoFisher Scientific, Shanghai, China). Cells were washed three times with the Hank's solution after adsorption for 2 h at 37 °C, and RNA was extracted with TRIzol reagent (Gibco BRL) as we described before[4]. Reverse transcription (RT) and

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PCR was performed by using the primers JV12 (5'-ATA CCA CTA TGA TGC AGA TTA-3', nucleotides location 4552-4572) and JV13 (5'-TCA TCA TCA CCA TAG AAA GAG-3', nucleotides location 4878-4858)[5]. RT and PCR were performed using a Qiagen OneStep RT-PCR kit. The thermal conditions for NoV-specific one-step RT-PCR were 50 °C for 30 min and 95 °C for 15 min, 40 cycles at 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 45 s, followed by a final extension of 72 °C for 10 min. Amplicons were separated by agarose gel electrophoresis with size markers and visualized by ethidium bromide fluorescence. Neutralization was determined by the absence of detectable GII.4 NoV RNA in the inoculated cell culture. A negative serum control, virus control, and uninoculated cell control were always processed for the detection of GII.4 NoV RNA at the same time for each assay.

The RT-PCR assay for GII.4 NoV RNA detection was evaluated based on the serially diluted virus stock suspensions (starting from 1:10 diluted in PBS). As described above, the serially diluted viruses were inoculated onto a cell monolayer of HIEC6 and adsorption for 2 h at 37 °C. After washing three times, viral RNA was extracted and RT-PCR was carried out. The results showed that the limit of NoV RNA detection in the present assay was 1500 copies/mL. As expected, the viral genome was undetecable in HIEC6 without NoV infection.

The sensitivity of the PCR-based rapid seroneutralization assay was tested with 26 acute-phase sera and 26 convalescent-phase sera from patients diagnosed with GII.4 NoV infection. As the results shown, all of convalescent-phase serum samples could neutralize virus *in vitro* at 1:10 dilution. The viral genome was undetectable immediately after washing at the end of the inoculation step. Moreover, the NoV genome remained undetectable while the cell culture was maintained for at least 10 d. However, all of the acute-phase sera failed to neutralize the virus infection *in vitro*. An example of an RT-PCR used to identify NoV rapid seroneutralization assay was shown in Figure 1.

The specificity of the PCR-based rapid seroneutralization assay was examined with 40 serum samples collected from healthy controls. There was one serum sample that repeatedly showed neutralization of virus. A possible explanation for this subject with neutralizing antibody might be that the individual was presently asymptomatically infected by NoV before or perhaps had a recent NoV infection. China is an endemic region of NoV infection with more than 90% of individual have been infected by NoV[6-7]. For the other 39 serum samples, the NoV RNA was always detected by RT-PCR at each step of the cell culture.

Enzyme-linked immunosorbent assays (ELISA) for anti-NoV IgG detection based on the virus-like particles of NoV has been widely used in the epidemiological investigations[8]. When the samples collected for this study were tested by ELISA (Yuanxiang Biological, Nanjing, China) for anti-NoV IgG antibody, 25 of 26 convalescent-phase serum samples were positive for anti-NoV IgG. The sensitivity was 96.2%, which was similar to the results obtained from the PCR-based seroneutralization assay. Among 40 serum samples from healthy controls, 8 samples showed anti-NoV IgG positive. The specificity is 80.0%, which was significantly lower than the results from the PCR-based seroneutralization assay (39/40, 97.5%) (χ^2=4.51, P<0.05) (Figure 2). However, the present ELISA assay is not specific for GII.4 NoV antibody detection and it can pick up total antibody to GII.4 NoV other than neutralizing antibody. Therefore, more studies should be performed to investigate these issues in future.

The cell culture for NoV replication provided us an opportunity to develop a simple and rapid neutralization assay *in vitro*. However, the lack of a cytopathic effect and the low level of NoV replication (the number of virions inoculated was only multiplied

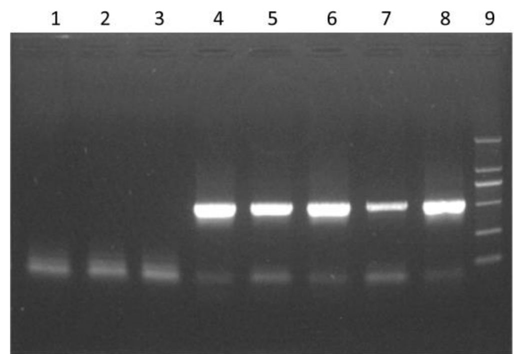

Figure 1. NoV RNA detected by PCR as marker for PCR-based seroneutralization assay *in vitro*. Lane 1, uninfected cell control; lane 2-3, virus with convalescent-phase serum at dilution of 1:10; lane 4-5, virus with acute-phase serum at dilution of 1:10; lane 6-7, virus with healthy controle serum at dilution of 1:10; lane 8: virus control; lane 9, molecular size marker.

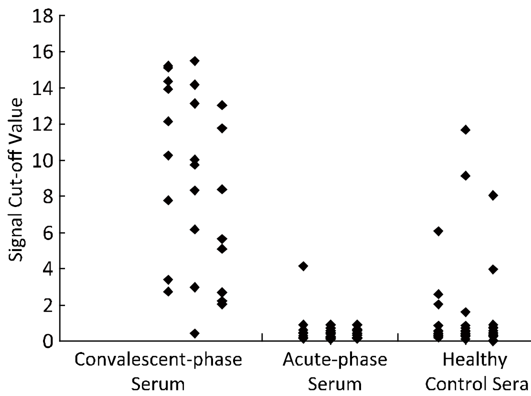

Figure 2. Distribution of NoV VLP-based ELISA reactivities in convalescent-phase serum samples, acute-phase serum samples and healthy controls serum samples, respectively. Horizontal line indicates the S/CO (signal cut-off value) of 1.

by 10^2) oblige us to use the sensitive RT-PCR to detect the presence of virus and to determine the neutralization effects in cell culture. As the results shown, the sensitivity of the present PCR-based rapid seroneutralization assay was 100%, which has a good correlation with the results of ELISA. However, the significant difference in the specificity was observed between the PCR-based seroneutralization assay (97.5%) and ELISA (80%). In the previous studies, very similar *in vitro* neutralization assays were developed to study the neutralizing antibody response to the hepatitis C virus (HCV) and hepatitis E virus (HEV) infection[9-10]. These assays are based on using cell surface receptors to specifically capture the virus, and antibody neutralization activity is assessed by demonstrating the absence of detectable virus genome in inoculated cells. Moreover, the PCR-based seroneutralization assays for HCV and HEV appear to be a more specific and more sensitive test than ELISA[9-10]. Taking together, the present results demonstrated that the new PCR-based seroneutralization assay is a reliable *in vitro* approach to evaluate the neutralizing antibody in anti-NoV serum specimens and is an efficient and fast alternative to *in vivo* animal models.

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We have no conflict of interest concerning this study.

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