Short Communication

TITRATION OF VARICELLA-ZOSTER VIRUS DNA IN THROAT SWABS FROM VARICELLA PATIENTS BY COMBINED USE OF PCR AND MICROPLATE HYBRIDIZATION

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SUMMARY: We devised a simple procedure for titration of varicella-zoster virus (VZV) DNA in throat swabs from varicella patients. DNA which was extracted from throat swabs, together with known copy numbers of a cloned VZV DNA fragment, were 10-fold serially diluted and used as template in PCR. The PCR products, after heat denaturation, again serially diluted in 1.5 M NaCl and adsorbed to microplate wells. Then, biotin-labeled DNA probes were hybridized with the immobilized DNA. The hybridization signal was produced by streptavidin-conjugated β-galactosidase and a fluorogenic enzyme substrate. By comparing the titration curves of a clinical specimen with those of the cloned fragment, of which detection limit was about 10 copies, we estimated the copy numbers of VZV DNA in the specimen. With this technique, we evaluated the degree of potential contagiousness of the patient along the course of infection: we found that varicella patients possessed highest quantity of VZV DNA in the throat on the first day of illness.
Varicella, which is caused by primary infection with varicella-zoster virus (VZV), is a highly contagious disease. Epidemiological data implicate airborne transmission of the disease, and thus the oropharynx of the patient is considered to be the main site of virus dissemination. Outbreaks of airborne varicella in hospitals have been reported (1,2). By unknown reasons, however, isolation of VZV in tissue cultures from throat swabs is very difficult (3).

After the advent of the PCR technology, researchers have re-investigated this issue and shown the presence of VZV DNA in the throat of varicella patients (4-6). VZV DNA was detected even from hospital air samples (7). No information has so far been available, however, on the amount of VZV DNA in the throat.

In this study, we quantified by a simple procedure, the combined use of PCR and microplate hybridization (8), VZV DNA in the throat swabs collected serially from VZV-infected children, and evaluated the potential infectiveness of the children during infection.

Throat swabs from the children were immersed into 1 ml of sterile phosphate-buffered saline, then frozen at −70 C until used. The thawed suspensions, after removal of the swabs, were centrifuged at 18,000 × g for 2 hr. The pellets were treated with 0.4 ml of 0.6% sodium dodecyl sulfate and 200 μg per ml of proteinase K in NTE buffer [0.1 M NaCl, Tris-HCl (pH 8.0), 10 mM EDTA] at 37 C for 3 hr. DNA extracted with phenol and chloroform was precipitated with ethanol from the aqueous phase and dissolved in 100 μl of NTE. Three dilutions (100, 10-1, and 10-2) of the DNA specimens, 10 μl each, were used in PCR. As template standards, 105, 104, 103, 102, and 101 copies per 10 μl of purified EcoRI D fragment (12.9 kbp) of VZV DNA (9) were also used in PCR.

PCR was carried out as previously described (8). DNA-DNA hybridization was carried out in microplate wells as previously described (8,10). In brief, the PCR products were treated twice with phenol and once with chloroform to remove completely mineral oil. DNA was precipitated with ethanol and dissolved in 100 μl of NTE. After heat denaturation, the DNA was 10-fold serially diluted from 1:5 to 1:5,000 in 100 μl-volumes with 1.5 M NaCl in microplate wells. The microplate was then incubated for 2 hr at 37 C to immobilize the DNA. After washing the wells, biotin-labeled probes were hybridized at 50 C overnight at 50% formamide with the solid-phase DNA. To detect the hybridization signal, a combination of streptavidin-conjugated β-galactosidase and 0.2 mM 4-methylumbelliferyl-β-D-galactoside was used. Fluorescence units (FU) of the enzyme reaction product were determined on a fluorometric microplate reader. Titration curves (FU versus template dilution) of both the standard and specimens at four

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Fig. 1. An example of VZV DNA titration. Titration curves of specimens and the template standard were drawn after each run of PCR. The curves of a specimen which covered the 100-1,000 FU range were chosen. By comparing these curves with the standard curves at corresponding dilutions of the PCR products, the copy number of virus DNA in the specimen was calculated. For example, a $10^{-1.2}$ dilution of specimen A equaled to $10^{3.8}$ copies per 10 µl; that is, undiluted specimen A contained $10^{5.0}$ copies per 10 µl ($10^{6.0}$ copies per swab). FU stands for fluorescence unit.

dilutions of PCR products were drawn. By comparing the curves of a specimen with the standard curves, the copy number of virus DNA in the specimen was calculated; an example is shown in Fig. 1.

Then, we studied the time course of VZV DNA titers in the throat swabs of varicella patients after the rash appearance. We followed five varicella children from the first day of illness to the 9th day. The results are shown in Fig. 2. Although varied among individuals, the DNA titers were the highest on the first day, with a gradual decline thereafter.

Next, we quantified also the DNA titers in the incubation period before the appearance of rash. We followed two contact cases of varicella, infected through intrafamilial transmission. Figure 3 shows the results. The VZV DNA remained in low titers for a limited time in the incubation period, then it abruptly increased to high titers coinciding with the appearance of rash.
Ozaki et al. (4) investigated by PCR the presence of VZV DNA in the throat of varicella patients; they detected the DNA in 26% of the swabs taken during the incubation period, and in 100% of those taken during the first 3 days after the onset of rash. In the present study, we further clarified that the amount of VZV DNA in the throat was the largest on the day of rash appearance, and that the levels in the incubation period were >100-fold lower than the highest levels. The primary site of VZV replication is considered to be the regional lymph nodes of the upper respiratory tract, the secondary site the liver and spleen, and the tertiary site the skin (11). Our finding indicates that somewhere in the oropharynx is also the tertiary site.

There is also epidemiological evidence showing that varicella patients are contagious even before their rash is observed (12,13). However, the duration of the pre-rash contagiousness may be very short, less than 24 hr (12). We did not detect VZV DNA 2 days before the appearance of rash in a contact case (household B, Fig. 3). Although a larger number of contact cases must be investigated for a definitive conclusion, varicella patients seem to be most contagious on the
Fig. 3. Quantity of VZV DNA in throat swabs of two contact cases of varicella in the incubation period. The contact cases were likely to be infected with VZV on the day when the index cases in the same household possessed the highest quantity of virus DNA in the oropharynx (the day of rash appearance).

first day of illness. They are not contagious in an earlier time of the incubation period when low-titered VZV DNA was detected; the presence of the DNA may reflect the primary VZV replication at the port of entry.

In the present study, to quantify VZV DNA, we carried out PCR with external standard. One of the most reliable procedure for genome quantification by PCR is the competitive amplification with internal standard (14). This method is ideal particularly for RT (reverse transcription)-PCR for RNA genomes. In contrast, the parallel amplification with external standard, as used in this study, is a simpler and less expensive procedure. We employed three dilutions per specimen for PCR, because some throat specimens contained polymerase-inhibiting activities when undiluted. We further employed four dilutions per each PCR product for hybridization to obtain appropriate titration curves. Therefore, we needed a total of 12 wells per specimen for hybridization. However, the microplate hybridization together with the use of a microplate fluorometer facilitated such testing. This technique may be also useful for quantification of other DNA viruses.
Part of this study was presented at the 31st Annual Meeting of the Japanese Society for Clinical Virology, held in Kanazawa, June 1990.

REFERENCES