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Comparison of infectious influenza A virus quantification methods employing immuno-staining

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ABSTRACT

Infections caused by highly variable influenza A viruses (IAVs) pose perpetual threat to humans as well as to animals. Their surveillance requires reliable methods for their qualitative and quantitative analysis. The most frequently utilized quantification method is the titration by plaque assay or 50% tissue culture infectious dose estimation by $TCID_{50}$. However, both methods are time-consuming. Moreover, some IAV strains form hardly visible plaques, and the evaluation of $TCID_{50}$ is subjective. Employment of immuno-staining into the classic protocols for plaque assay or $TCID_{50}$ assay enables to avoid these problems and moreover, shorten the time needed for reliable infectious virus quantification. Results obtained by these two alternatives of classic virus titration methods were compared to the newer rapid culture assay (RCA), where titration endpoint of infectious virus was estimated microscopically based on the immuno-staining of infected cells. In our analysis of compared methods, five different IAV strains of H1, H3 and H5 subtypes were used and results were statistically evaluated. We conclude that the RCA proved to be at least as reliable in assessment of infectious viral titer as plaque assay and $TCID_{50}$, considering the employed immuno-staining.

1. Introduction

Since the discovery of the first viruses, the quantification of infectious virus particles is almost essential in the field of virology. The infectious virus titer i.e. the concentration of infectious virus particles in sample is mostly determined by inoculation of serial dilutions of virus into susceptible cell cultures. The presence of infectious virus is monitored by different techniques (Flint et al., 2015). The first developed and ever since most used method for quantification of infectious IAV is plaque assay (Gaush and Smith, 1968). In this case IAV produces localized cytopathic effect (CPE) in tissue culture covered with solid overlay medium, which allows only radial cell-to-cell spread of IAVs in cell monolayer. One plaque represents the progeny of one infectious virus particle, which can be used to acquire the genetically homogeneous population of IAVs. The concentration of infectious virus is expressed as a number of plaque forming units per milliliter of sample (PFU/ml). However, some IAVs produce hard to count, microscopic or opaque plaques either due to undetached infected/dead cells in terms of fixation/staining method used, or due to inherent characteristic of particular IAV strain (Hatakeyama et al., 2005; Matrosovich et al., 2006). The time needed for development of visible plaques takes generally three to five days when the conventional crystal violet negative staining of plaques is used. The classic plaque assay is time-consuming and it can be often laborious to optimize conditions, because of special experimental requirements such as sample origin, viral strain, cell culture type and special additives. The inclusion of immuno-staining into this protocol made the plaque assay more easily applicable for different virus strains, avoiding the problems with plaque visibility (Matrosovich et al., 2006).

Another widely used method to quantify infectious virus particles is 50% tissue culture infective dose ($TCID_{50}$) assay (Klimov et al., 2012). The amount of infectious virus in sample is estimated from titration end-points as the highest dilution of sample that can cause cytopathic effect (CPE) in 50% parallels of tissue culture samples. The assay is generally performed on 96-well plate at least in four parallels per sample. Usually the CPE positive/negative wells are evaluated manually under the inverted microscope in three to five days. To avoid the subjective error of manual CPE evaluation, the media are often tested

Abbreviations: IAV, influenza A virus; CPE, cytopathic effect; $TCID_{50}$, 50% tissue culture infective dose; RCA, rapid culture assay; IVT, infectious virus titer; NP, nucleoprotein; MAb, monoclonal antibody; TCA, trichloroacetic acid; MDCK, Madin-Darby canine kidney; HA, hemagglutinin; NA, neuraminidase; IgG, immunoglobulin G; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; CC, cell control

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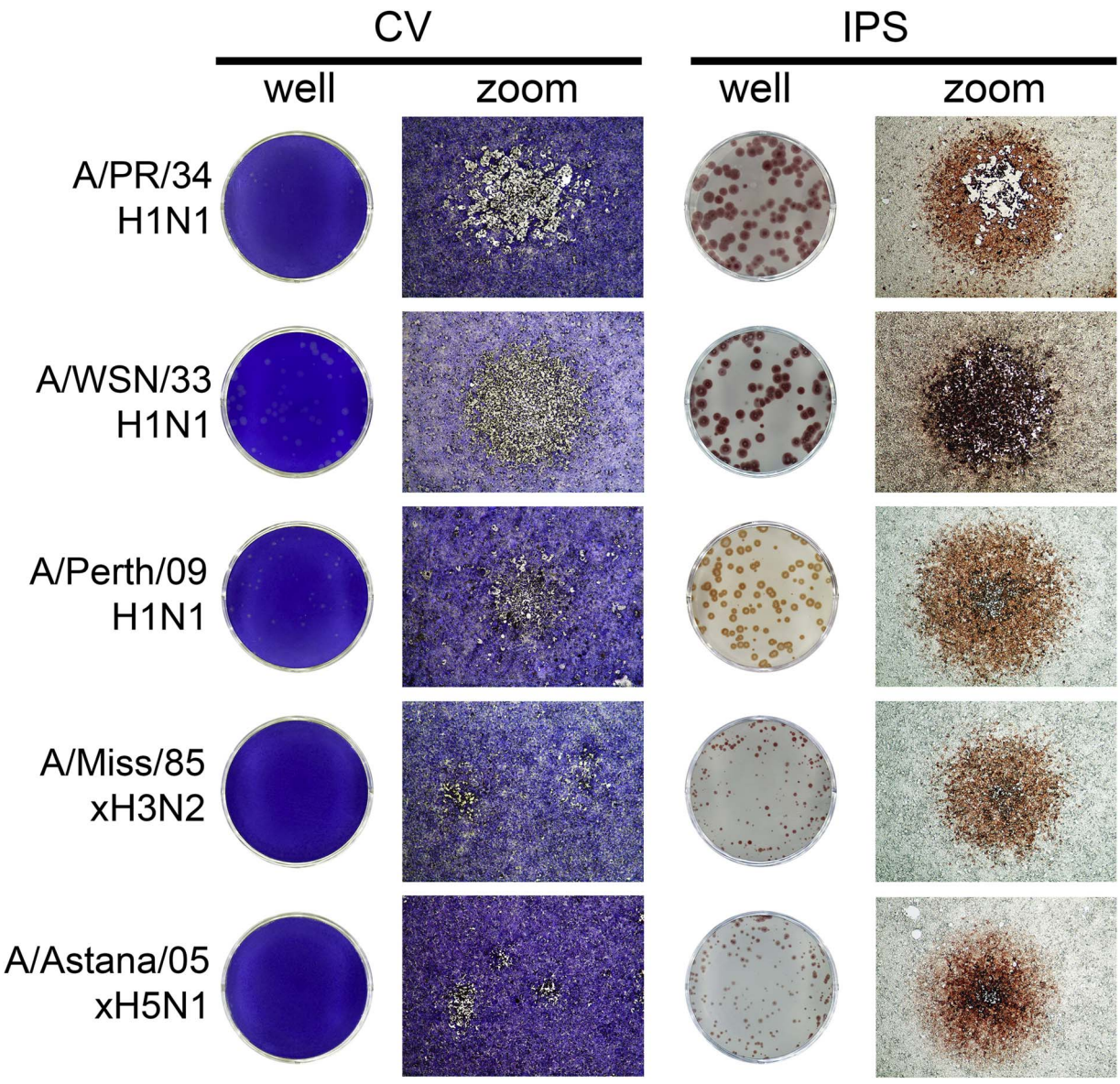

Fig. 1. An example of plaque assay titration of each examined virus strain on 6-well plate with microphotograph of plaque. Each titration with same virus strain and different staining method was carried out at the same time and the same dilution of the same aliquot sample was used to infect the cell monolayer. The virus strain name and subtype is depicted. The pictures were taken with Olympus E-600 camera and processed with Adobe Photoshop CS. CV – crystal violet, IPS – immuno-peroxidase staining. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

for virus presence by hemagglutination. For easier evaluation of results and its objectivity the immuno-staining was included also into this method. In this case the $TCID_{50}$ assay is finished on the second day in a similar way to enzyme-linked immunosorbent assay (ELISA) utilizing anti-NP MAb and soluble peroxidase substrate (LaBarre and Lowy, 2001; Myc et al., 1999). The infectious virus titer is calculated

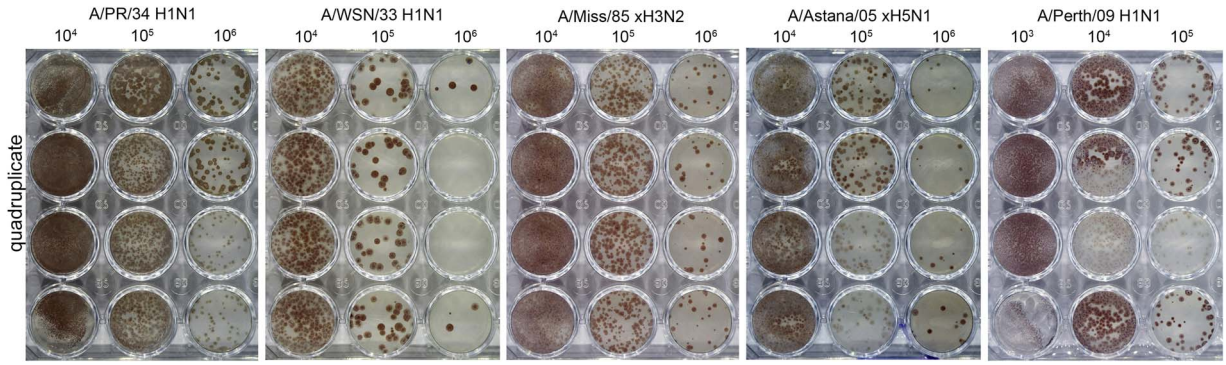

Fig. 2. Representative of plaque assay titration of each examined virus on 24-well plate, stained with precipitate-forming peroxidase substrate AEC. The strain name, subtype and dilution factors are indicated.