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Detection of influenza antigenic variants directly from clinical samples using polyclonal antibody based proximity ligation assays

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ABSTRACT

Identification of antigenic variants is the key to a successful influenza vaccination program. The empirical serological methods to determine influenza antigenic properties require viral propagation. Here a novel quantitative PCR-based antigenic characterization method using polyclonal antibody and proximity ligation assays, or so-called polyPLA, was developed and validated. This method can detect a viral titer that is less than 1000 $TCID_{50}$/mL. Not only can this method differentiate between different HA subtypes of influenza viruses but also effectively identify antigenic drift events within the same HA subtype of influenza viruses. Applications in H3N2 seasonal influenza data showed that the results from this novel method are consistent with those from the conventional serological assays. This method is not limited to the detection of antigenic variants in influenza but also other pathogens. It has the potential to be applied through a large-scale platform in disease surveillance requiring minimal biosafety and directly using clinical samples.

Introduction

The influenza virus is a perpetual threat to public health. Seasonal influenza infections are associated with thousands of deaths every year in the United States (Thompson et al., 2010). A worldwide pandemic could increase the death toll to millions in a short period of time. The hallmark of the influenza virus is antigenic variation, which comes in two forms: antigenic drift and antigenic shift, leading to the recurrence of influenza virus infections (Katz et al., 1987). Mutations in the hemagglutinin and neuraminidase glycoproteins cause antigenic drift. Meanwhile, antigenic shift is caused by the replacement of a new subtype of hemagglutinin and sometimes neuraminidase through genetic reassortment.

The influenza vaccine is the most viable option in counteracting and reducing the impact of influenza outbreaks (Harper et al., 2004). Since influenza viruses are continuously changing their antigenicity in order to escape the host immunity (Nobusawa and Nakajima, 1988; Webster et al., 1982), the vaccine strains need to be updated almost annually to obtain antigenic matches between the vaccine strain and the strain potentially causing future outbreaks (Ampofo et al., 2012; Gerdil, 2003). Identification of influenza antigenic variants is the key to a successful influenza vaccination program for both pandemic preparedness as well as seasonal influenza prevention and control (Katz et al., 1987).

Routinely, immunological tests, such as hemagglutination inhibition (HI) assays and microneutralization (MN) assays, have been relied upon to identify antigenic variants among the circulating strains (Medeiros et al., 2001). The HI assay is an experiment to measure how a test influenza antigen and a reference antigen (e.g. a current vaccine strain) match through the immunological reaction between the test antigen and the reference antiserum. This reference antiserum is usually generated in ferrets using the reference antigen. HI assays are limited due to their use of red blood cells (RBCs), e.g. turkey red blood cells, as indicators for the binding affinity of antigen and antiserum (Kendal et al., 1982). A higher interaction between antigen and antisera will lead to less hemagglutination of RBCs (Hirst, 1941). Compared to HI assays, MN assays seem to be more sensitive and specific but are much more time-consuming. Moreover, for influenza viruses requiring biosafety-level 3 (BSL-3) or higher, MN assays are difficult to perform (Grund et al., 2011). For this reason, HI assays have been one of the routine procedures used to identify influenza antigenic variants for vaccine strain selection while MN assays are generally used to validate the results from HI assays.

However, the data from HI assays are notoriously noisy, and HI experiments are affected by many factors. For example, RBCs used from different species and even variation in RBC sialic acid receptors

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can produce varied results (Medeiros et al., 2001). The data are subjective interpretations and the HI assays have difficulty in automating and standardizing operations. Minor antigenic variants within a heterogeneous population cannot be assessed by the serological method of HI (Patterson and Oxford, 1986). More importantly, mutations of the receptor binding site in HA (Nobusawa and Nakajima, 1988) (antigenic drift) are causing human seasonal H1N1 (Azzi et al., 1993; Morishita et al., 1993) and H3N2 influenza A viruses (Nobusawa et al., 2000) to lose the ability to bind to RBCs. For example, the mutations at residues 193, 196, 197, and 225 in the human epidemic H1N1 influenza A viruses in 1988 or later caused the loss of their abilities to agglutinate chicken RBCs due to four amino acid changes (Morishita et al., 1996). For H3N2 viruses, the Gly190Asp substitution has been correlated to the loss of the ability to agglutinate chicken erythrocytes (Cox and Bender, 1995; Fitch et al., 1997; Lindstrom et al., 1998, 1996; Medeiros et al., 2001; Mori et al., 1999; Nobusawa et al., 2000). Since 2000, human seasonal H1N1 and H3N2 influenza A viruses have been losing their binding abilities to turkey red blood cells (Medeiros et al., 2001; Oh et al., 2008). This may be attributed to a reduced affinity for sialic acid-linked receptors (particularly α2-6-linked receptors), which are at lower levels on chicken and turkey RBC compared to levels on guinea pig RBC (Medeiros et al., 2001; Oh et al., 2008). Consequently, a critical demand exists for the development of a red blood cell independent assay for influenza antigenic variation.

Proximity ligation assay utilizes quantitative real time PCR (qRT-PCR) for the detection of antigen–antibody interaction (Schlingemann et al., 2010). For this assay: (1) oligonucleotide-linked monoclonal antibodies are incubated with the analyte in question; (2) if in close proximity, the oligonucleotides can be ligated together; and (3) presence of analyte will be shown by amplification of ligated products with qRT-PCR. The assay reporter signal is dependent on a proximal and dual recognition of each target analyte providing high specificity (Fredriksson et al., 2007).

In this study, we developed a novel antigenic characterization method using polyclonal antibody-based proximity ligation assays (polyPLA). This method was found to be useful in detecting influenza antigenic variants in clinical samples.

Results

PolyPLA for influenza antigenic variant detection

PolyPLA quantifies the antibody antigen binding avidity using the amplification signals in quantitative PCR (qPCR) from the pairs of primers attached to a reference polyclonal antiserum. The first step of this experiment is to biotinylate a reference polyclonal antiserum (Fig. 1A), which will be then labeled with sodium azide-linked 5′ and 3′ oligonucleotides (Fig. 1B). The ligation efficiency will be assessed with qPCR. A labeled polyclonal antiserum with $\Delta Ct \geq 8.5$ in the ligation efficiency test is then incubated with a reference antigen (virus) or a testing antigen (Fig. 1C), followed by the proximity ligation of the two oligos (Fig. 1D). The antibody antigen binding avidity is quantified using the amplification signals ΔCt in qPCR (Fig. 1E). The ΔCt values among the polyclonal antisera and antigens can be compared to assess antigenic differences among these tested antigens. These ΔCt values can be viewed as similar to the serological titers, such as HI and neutralization titers, from conventional serological assays (Fig. 1F).

To make the ΔCt values comparable across reference sera, we have to ensure that the testing antigens have the same quantities across quantification assays. In HI assays, we usually standardize the antigens to be 4 units of hemaglutinnation titer before HI; in neutralization assays, we usually standardize antigen quantities using $TCID_{50}$ (2013). In this assay, we use the quantities of nucleoproteins (NPs) to normalize the amount of viruses in the analyses. For data consistency, we used a monoclonal antibody targeting conserved regions of NPs in the proximity ligation assay (Schlingemann et al., 2010). Thus, for a testing antigen, the polyPLA units were normalized by its ΔCt values for polyclonal antiserum (polyΔCt) with its ΔCt values for monoclonal antibody against NP (monoΔCt) (Fig. 1G).

Viral particles must be completely lysed to release NPs and allow for an accurate measure of these protein quantities. We compared two commonly used methods for viral lysis: freeze/thaw and treatment with lysis buffer. The results showed that lysis buffer treated virus had a significantly higher ΔCt value of 7.46 (± 0.45) for A/Sydney/05/1997 (SY/05), $p < 0.05$ (Fig. 2) compared to the freeze/thaw method of viral lysis. However, for A/Sichuan/2/1987 (SI/2), lysis buffer treated virus did not have a significantly different ΔCt value compared to that of the freeze/thaw method of viral lysis. In the following assays, all the samples used in normalization were treated with lysis buffer.

HA specific IgG predominates polyclonal antisera

PolyPLA quantifies the interactions between influenza viral proteins and all IgG present in the polyclonal antisera. To assess the impacts of NA and other internal proteins on polyPLA, we constructed three reassortants between SY/05 and PR8, including SY/05xPR8 (H3N2), SY/05xPR8(H3N1), and SY/05xPR8(H1N2). The signals from NPC were used as the control to calculate ΔCt value from proximity ligation assays. Our results showed that SY/05, SY/05xPR8(H3N2), and SY/05xPR8(H3N1) had ΔCt values of 5.40(± 0.74), 5.67(± 0.17), and 5.26(± 0.34), respectively (Fig. 3). The ΔCt values from PR8 and the reassortant SY/05xPR8(H1N2) were negligible.

Viral quantities are linearly correlated with ΔCt values

To assess the sensitivity of polyPLA, we performed PLA on influenza A viruses with serial dilutions. Regression analyses demonstrated that the polyΔCt values are linearly correlated with the influenza viral quantities, with Pearson's coefficient $R = 0.98$ for the testing strain SY/05 ($p < 0.001$) (Fig. 4). The cutoff ΔCt value 3.00 was equivalent to 4.90×10^4 $TCID_{50}$/mL against its homologous polyclonal antibodies. Similarly, the mono-ΔCt values were also linearly correlated with HA titers, and the R was 0.92 for SY/05 ($p < 0.001$). The cutoff ΔCt value was equivalent to 9.80×10^4 $TCID_{50}$/mL against the NP-specific monoclonal antibody. Similar linear correlations were also observed in A/Johannesburg/33/1994(H3N2) (JO/33) and A/Nanchang/933/1995 (H3N2) (NA/933) (data not shown). Linear correlation between viral quantities and ΔCt allows us to normalize the viral titers by using a simple equation such as $a \times (\text{polyΔCt} - \text{monoΔCt}) + b$, where a and b are constant parameters. This normalization method enables us to compare the antigenic properties between the testing antigens (viruses) without justifying the viral quantities before measuring polyΔCt, having been used in HI and neutralization assays to ensure the equivalency of the viral quantities before assays.

Sensitivities of polyPLA

To test the sensitivity of polyPLA, we evaluated the viral loads from nasal swabs collected from ferrets infected with A/swine/K6/2011 (H6N6). After only the first day of infection, a polyΔCt titer of 3.20 (± 0.06, standard deviation) was obtained, corresponding to a $TCID_{50}$ titer of 1.00×10^3 for the infected ferret (Fig. 5). After two days of infection, a polyΔCt value of 5.19 (± 0.06) was obtained, corresponding to a $TCID_{50}$ titer of 1.00×10^4. A higher polyΔCt titer corresponded to a higher $TCID_{50}$ titer among the nasal wash samples post-infection. All the samples collected from the control ferrets had polyΔCt titers of less than 3.00, and no viruses were recovered from these control ferrets (Fig. 5). Thus, this method is sensitive sufficiently to detect not