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# ViroSpot microneutralization assay for antigenic characterization of human influenza viruses

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ABSTRACT

The hemagglutination inhibition (HI) assay has been used for the antigenic characterization of influenza viruses for decades. However, the majority of recent seasonal influenza A viruses of the H3N2 subtype has lost the capacity to agglutinate erythrocytes of various species. The hemagglutination (HA) activity of other A(H3N2) strains is generally sensitive to the action of the neuraminidase inhibitor oseltamivir, which indicates that the neuraminidase and not the hemagglutinin is responsible for the HA activity. These findings complicate the antigenic characterization and selection of A(H3N2) vaccine strains, calling for alternative antigenic characterization assays. Here we describe the development and use of the ViroSpot microneutralization (MN) assay as a reliable and robust alternative for the HI assay. Serum neutralization of influenza A(H3N2) reference virus strains and epidemic isolates was determined by automated readout of immunostained cell monolayers, in a format designed to minimize the influence of infectious virus doses on serum neutralization titers. Neutralization of infection was largely independent from rates of viral replication and cell-to-cell transmission, facilitating the comparison of different virus isolates. Other advantages of the ViroSpot MN assay include its relative insensitivity to variation in test dose of infectious virus, automated capture and analyses of residual infection patterns, and compatibility with standardized large scale analyses. Using this assay, a number of epidemic influenza A(H3N2) strains that failed to agglutinate erythrocytes, were readily characterized antigenically.


## 1. Introduction

Influenza viruses are an important cause of respiratory tract infections. Antigenic variation of the hemagglutinin (HA) allows these viruses to evade recognition by virus-specific neutralizing antibodies induced upon previous infection or vaccinations, and cause epidemic outbreaks annually.

Current influenza epidemics are caused by influenza A viruses of the H3N2 and H1N1 subtypes and influenza B viruses. The influenza A viruses are descendants of the pandemic strains of 1968 and 2009 respectively [1]. Two antigenically distinct lineages of influenza B viruses co-circulate since the mid 80s, spurring the development of quadrivalent influenza vaccines, containing components of both influenza A subtypes and both influenza B lineages [2].

The efficacy of influenza vaccines depends on the antigenic match between the vaccine and circulating strains. Because of the antigenic drift of influenza viruses, vaccine strains are annually selected, and when necessary updated, to match epidemic strains predicted to circulate in the following season. However, mismatch may occur, as during the 2014/2015 influenza season, resulting in reduced vaccine effectiveness [3–5].

The hemagglutination inhibition (HI) assay has been used for the antigenic characterization of influenza viruses, for decades. For this assay, mono-specific antisera against Reference and representative epidemic influenza strains are produced in ferrets upon experimental infection. Serial serum dilutions are incubated with a standard amount of the respective viruses and the highest dilution that still prevents the virus from agglutinating erythrocytes is recorded. This method proved instrumental for influenza vaccine strain selection and update for many years [1,6]. Although the method is still successful for the antigenic characterization of influenza A(H1N1) and B-viruses, problems have arisen with that of A (H3N2) viruses. This is mainly caused by evolutionary changes in

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the A(H3N2) HA that resulted in the loss of capacity to agglutinate chicken or turkey erythrocytes. As an alternative, erythrocytes of mammalian species, like human type O or guinea pig erythrocytes, have been used. However, although A(H3N2) viruses displayed some HA activity with mammalian erythrocytes, in many cases this activity proved to be sensitive to the addition of the neuraminidase (NA) inhibitor oseltamivir, indicating that NA, and not HA, was responsible for binding to the erythrocytes and mediated HA activity [7].

These issues have raised interest in alternative assays that can be used for the antigenic characterization of influenza viruses, such as virus neutralization (VN) assays [8,9]. In these assays, ideally a standard number of infectious units (e.g. 100 50% tissue culture infectious dose (TCID50)) is incubated with serial dilutions of serum samples. Residual non-neutralized virus is detected by inoculation of susceptible cells, in most cases Madin-Darby canine kidney (MDCK) cells. The infection of these cells is assessed by monitoring the development of cytopathic changes, or by detecting viral protein synthesis using enzyme-linked immunosorbent assay (ELISA) [10,11] or immunostaining of virus-infected cells [8].

Here we describe the ViroSpot microneutralization (MN) assay as a novel assay with favorable properties and its use for the antigenic characterization of epidemic A(H3N2) influenza viruses, which may aid vaccine strain selection.

## 2. Materials and methods

### 2.1. Cells

MDCK cells were cultured in Eagle minimal essential medium (EMEM; Lonza, Verviers, Belgium) containing 20 mM HEPES buffer (Lonza), 0.075% sodium bicarbonate solution (Lonza), 2 mM L-glutamine (Lonza), 100 IU/ml penicillin-100 μg/ml streptomycin (Lonza), referred to as complete medium (CM), supplemented with 10% fetal bovine serum (FBS; Bodinco BV, Alkmaar, The Netherlands). The cells were passaged to new culture flasks twice weekly. Two days before inoculation, the cells were seeded at a concentration of about $10^4$/well in flat-bottom 96-well tissue culture-treated microplates (Greiner Bio-One, Alphen aan den Rijn, The Netherlands).

### 2.2. Viruses and serum samples

Epidemic influenza viruses that were isolated in the Netherlands were obtained from the repository of the Dutch National Influenza Center. Ref. virus strains were provided by The Francis Crick Institute, Mill Hill Laboratory, The Crick Worldwide Influenza Center and The National Institute for Biological Standards and Control (NIBSC), London. All viruses were propagated in MDCK cells cultured in infection medium (IM; complete medium without FBS, and with 3 μg/ml tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich, Zwijndrecht, The Netherlands)). Infectious titers of the virus stocks were determined in MDCK cells as described previously [12], or with minor modifications using half-log or five-fold dilutions and the ViroSpot immunostaining described herein. In brief, confluent monolayers of MDCK cells were inoculated with replicate (n = 4) serial 3.16-fold or 5-fold dilutions of virus stocks in IM in 96-well microtiter plates. After 90 min at 35 °C in a humidified 5% $CO_2$ incubator, the inocula were removed and the cells washed with IM. The cells were then cultured for 2 days in a humidified incubator at 35 °C and 5% $CO_2$. On each plate wells with uninfected cells were included as negative controls. As positive controls, cells infected with one of at least two reference virus strains, were included in each experiment.

Post-infection Ref. sera were produced in ferrets essentially as described previously [13]. Before and two weeks after intranasal inoculation, blood was drawn and serum collected. A sheep high titer standard serum directed to influenza virus A/Texas/50/2012 was obtained from NIBSC, and used for initial set-up of the assay.

### 2.3. ViroSpot microneutralization (MN) assay

Serum samples were pretreated with receptor destroying enzyme (RDE) by incubating 100 μl of serum with 500 μl of an in-house produced filtrate of *Vibrio cholera* for 16 h at 37 °C, and heat-inactivated for one hour at 56 °C. The pretreated serum was diluted to 1 ml with IM for a 1:10 dilution. Sera showing a high homologous titer were further diluted (e.g. to 40× or 320×) to ensure that the dilution representing the titer was detected on the test plate. Subsequently, serial dilutions were made with IM in round-bottom plates and 60 μl of each dilution was mixed with 60 μl of virus suspension at the desired concentration of infectious units (e.g. 100 TCID50/50 μl, as calculated from TCID50 stock titers that were determined by using serial five-fold dilutions). After one hour at 35 °C, 100 μl of the mixtures was transferred to MDCK cells that were washed once with phosphate buffered saline (PBS) and once with IM. After an incubation for 90 min at 35 °C, the virus-antibody mixtures were removed, cells were washed once with IM and overlayed with 1.6% carboxymethylcellulose (medium viscosity, CMC, Sigma) in CM with 2 μg/ml modified trypsin (TPCK-treated), and cultured for 2 days at 35 °C and 5% $CO_2$. CMC is used to restrict viral spread in the cell monolayers. In each assay, uninfected control cells were included as well as virus controls without serum incubation. The virus test dose was confirmed by back titration. To this end, serial half-log or five-fold dilutions were used to inoculate MDCK cells in duplicates, and were processed in parallel to the serum assay plates.

Next, the cells were washed at least twice with PBS to remove the CMC overlay and 100 μl 10% formalin (Sigma) was added to the wells. After 15 min at room temperature, or at least 16 h at 4 °C, or up to 30 days at −20 °C, the formalin was removed and plates were washed once with PBS. The variable formalin fixation times allow for completion of small-scale experiments with a few plates on the day of fixation on the one hand, and for processing large batches of plates within the timeframe of several days or weeks on the other hand. A volume of 100 μl of 0.5% Triton X-100 (Applichem GmbH, Darmstadt, Germany) was added and the plates were incubated for 10 min at room temperature. After washing the plates with PBS, ViroSpot immunostaining was performed by incubating the formalin-fixed and permeabilized cells for one hour at room temperature with 50 μl of a mouse monoclonal antibody (HB65; EVL, Woerden, The Netherlands) directed against the viral nucleoprotein (NP), followed by a one hour incubation at room temperature with 50 μl of a horseradish peroxidase-labeled goat anti mouse immunoglobulin preparation (GAM-HRPO, Invitrogen, Foster city, CA). Antibody reagent dilutions were made in PBS containing 2% (w/v) Skimmed Milk Powder (Sigma), and optimized for each reagent lot. HB65 (2 mg/ml) and GAM-HRPO (1 mg/ml) were used at 1/20,000 and 1/10,000 dilutions respectively. After each incubation step, the plates were washed three times with PBS containing 0.05% Tween-20. After the last washing step, 50 μl of True-Blue substrate (KPL, Gaitherburg, Maryland) was added per well and incubated for 10 min at room temperature. The plates were washed four times with distilled water, the last time for 30 min. After drying, the plates were submitted to automated image capture using a Series 6 ImmunoSpot Image Analyzer (CTL ImmunoSpot, Cleveland OH, USA) to quantitate the percentage well area covered by spots of infected cells. The percentage inhibition was calculated according to the following formula:

**Table 1**
Microneutralization (MN) assay formats: comparison of similarities and differences that impact basic virological principles and assay robustness of WHO MN and ViroSpot MN.

| | Process | WHO MN | ViroSpot MN |
|---|---|---|---|
| Infectious virus titration | Concentration of virus stocks (infectious units) | 1 unit = the dilution factor required for 2× higher signal than uninfected cells in 50% of cultures | 1 unit = the dilution factor required to infect 50% of cultures ($TCID_{50}$) |
| | Incubation temperature and period | 18–20 h at 37 °C; irrespective of virus replication kinetics | Until all infected wells become positive; 1–2 days at 35 °C, depending on replication kinetics |
| Test virus preparation | Dilution factor | To obtain 100 infectious units/well | To obtain 100 $TCID_{50}$/well |
| | Target concentration ($TCID_{50}$/well) | Depends on kinetics of virus replication and cell-to-cell transmission | 100 |
| | Expected range around 100 ($TCID_{50}$/well) | Unknown | 20–500 |
| Neutralization method | Mix virus and serum | 60 min | 60 min |
| | Indicator cells (MDCK) to monitor infection/neutralization | Add cells to virus/serum mixtures | Add virus/serum mixtures to confluent monolayer of cells |
| | Replace inoculum with overlay medium | No | Yes (1.6% CMC after 90 min) |
| | Incubation | 18–20 h at 37 °C (INF A) | 1–2 days at 35 °C |
| | Fixation of cells | Acetone | Formalin |
| | Detection of virus propagation | NP-ELISA (soluble TMB) | NP-immunostaining (precipitating TMB) |
| | Signal | OD450-620 nm | Well area covered |
| | Neutralization cut-off | ⩽50% | ⩽90% |
| | Rationale for cut-off | Evidence of neutralization | Exclude variability around 100% |
| Neutralization of different viruses | Amount of test virus depends on replications kinetics | Yes | No |
| | Effect of variation in $TCID_{50}$ on the amount of serum required for neutralization | Variable | Low |

$$100 * (1 - (X - CC)/(VC - CC))$$

where

X is the value of well area covered by spots of infected cells (% WAC) in wells inoculated with virus/serum mixtures.
CC is the average of %WAC in the cell control wells (no virus).
VC is the average of %WAC in the virus-control wells (no serum).
Inhibition ⩾90% was considered positive for neutralization.

When viruses were back titrated, the number of infectious units were determined by counting immunostained plaque forming units (PFU) at dilutions that resulted in easily countable number of plaques per well (approximately between 10 and 60). The test dose of virus was considered acceptable if it was between 14 and 345 PFU/well, which corresponds to 20 and 500 TCID50/well and is ± one dilution step around the target dose of 100 TCID50/well when 5-fold dilutions were used for stock virus titration.

### 2.4. WHO MN assay

Previously, a MN assay was described to circumvent some of the HI assay-related issues [10]. This MN assay has been adopted by the WHO for serologic analyses. In the present study on antigenic typing, the assay was carried out essentially as described in the WHO Manual for the laboratory diagnosis and virological surveillance of influenza [11]. The same NP-specific monoclonal antibody and GAM-HRPO were used as described above for ViroSpot immunostaining, but in a volume of 200 µl of a 1/3,000 and 1/30,000, respectively, for use with TMB substrate (Sigma). An overview of characteristics and differences of the WHO MN and ViroSpot MN assay is presented in Table 1.

### 2.5. HI assay

After treatment with RDE from in-house cholera filtrate and heat-inactivation at 56 °C, the sera were tested for the presence of anti-HA antibodies by HI assay using standard protocols for serological diagnosis [11], with modifications for antigenic typing. In brief, 50 µl of serially diluted pretreated serum samples were incubated with four hemagglutinating units of virus in the presence of Oseltamivir carboxylate (Roche, Basel, Switzerland; end concentration 20nM). After 30 min at 37 °C, 25 µl of 1% guinea pig erythrocyte (Bio Trading, Mijdrecht, The Netherlands) suspension in PBS containing 1% BSA (Invitrogen) was added. Agglutination patterns were read by two technicians independently after an incubation period of 2 h at 4 °C.

## 3. Results

### 3.1. Receptor destroying enzyme

During earlier attempts to develop the Virospot (VS) MN assay, some pre-infection ferret sera showed non-specific neutralization of virus (Fig. 1). Pre-treatment of serum with a filtrate of *Vibrio cholera* as a source of RDE, successfully prevented this non-specific non-antibody-mediated inhibition, by effectively removing HA receptor molecules from serum components. Post-infection serum retained its capacity to neutralize the virus with titers similar to those of untreated serum samples. An example of the effect of RDE treatment is shown in Fig. 1. Similar findings were obtained with five other ferret serum pairs obtained with 3 different viruses (X-187, IVR-165 and X223A). Thus, RDE treatment of serum samples is recommended for use in VS MN assay to prevent non-specific neutralization of virus.

### 3.2. Sensitivity of ViroSpot and WHO MN assays to variations in quantity of input virus

To allow meaningful and reliable antigenic comparison of different virus strains, it is imperative that a standardized quantity of input virus is used for each of the viruses tested. Indeed, the level of neutralization of each virus tested by a reference serum depends on the quantity of infectious units present during the course of the antigenic typing assay. For the HI assay, this is relatively easily achieved by using 4 hemagglutinating units of virus. For MN assays, the infectious virus titer of each virus stock needs to be determined. Assessment of infectious virus titers is subject to variability, which is generally accepted within ±1 dilution step from the mean titer. Following a single experiment, the true titer

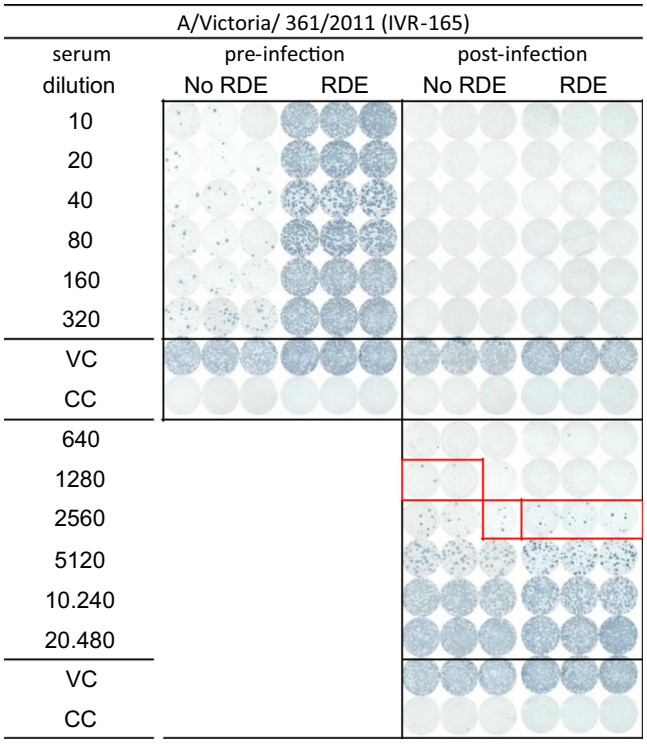


**Fig. 1.** Receptor destroying enzyme (RDE) removes non-specific inhibition from pre- and post-infection sera obtained from a ferret experimentally infected with influenza virus A/Victoria/361/2011 (IVR-165). Sera were tested in triplicate. Red box: Highest dilution of post-infection ferret serum showing ⩾ 90% inhibition. VC: virus control; CC: cell control.

of a virus stock may therefore be over- or underestimated by up to one dilution step. Virus test doses aiming at 100 TCID50 are therefore expected to range between 20 and 500 TCID50, if the original stock titration was based on a five-fold dilution series.

To compare the effect of differences in input virus quantity on WHO MN and Virospot MN assay measurements, we performed both assays with influenza virus A/Texas/50/2012 using 10–600 TCID50 per well and a sheep antiserum raised against this virus. As shown in Table 2, the geometric mean titers (GMT) measured in the WHO MN assays ranged from 45,255 when 10 TCID50 of virus was used to 8,000 when ⩾300 TCID50 was used. Based on the WHO MN criteria for back titration values, only the 30 and 100 TCID50 cultures lay within the acceptable range. For 300 TCID50, the WHO MN titer was 8,000, which is already 4-fold lower than observed with a 10-fold higher virus concentration. In contrast, the GMT MN titers measured in the ViroSpot MN assay differed no more than 2-fold using the same virus suspensions ranging from 10 up to 450 TCID50 (Table 2 and Fig. 2). This shows that ViroSpot MN assay results are less sensitive to variability in input test virus concentrations. Erroneous interpretation of virus concentration difference as an antigenic difference therefore is less likely.

### 3.3. Antigenic characterization of reference strains

We compared the performance of both MN assays for the antigenic characterization of influenza A(H3N2) viruses using selected Ref. strains and ferret sera raised against three vaccine strains, A/Victoria/210/2009 (X-187), A/Victoria/361/2011 (IVR-165) and A/Texas/50/2012 (X-223A). The input virus dose was aimed at 100 TCID50. Back-titration of all Ref. and vaccine strains ranged between 49 and 293 TCID50, within the expected ±1 dilution step of 100 TCID50. As shown in Table 3, WHO MN and ViroSpot MN titers correlated reasonably well. Also, the identification of antigenic match with the vaccine strains was in general comparable between the two MN assays and similar to that of HI assays as deduced from the WHO consultation reports (http://www.crick.ac.uk/research/world-wide-influenza-Center).

However, the WHO MN assay did not produce antibody titers of vaccine strain-specific antisera for three strains, A/Iowa/19/2010 (for one out of two sera), A/Perth/16/2009 and A/Hong Kong/146/2013, because of unclear neutralization patterns due to a combination of low virus signal and high serum background at low dilutions (Table 3). For these viruses, the WHO MN virus control values were the lowest among the virus strains tested. The respective virus test doses were 131, 142 and 49 TCID50/well, as determined by back titration of input virus used in the ViroSpot MN assay, within the expected range for targeted inocula of 100 TCID50. Lower signals in the WHO MN assay could have resulted from lower infection and replication efficiency. This may be compensated by testing higher virus concentrations, but this is contrary to the aim of comparing antigenic differences at similar virus concentrations. The low virus signal combined with elevated background ELISA signals at low serum dilutions resulted in false positives in the WHO MN assay. The Virospot MN assay using the same virus suspensions and serum dilutions provided results, since serum background signals were inherently lower and did not increase for lower serum dilutions. While the serum is not removed in the WHO MN assay, contributing to higher optical density ELISA signal, serum and virus are removed following a brief inoculation period in the VS MN assay. In addition, the WHO MN assay readout is the optical density of a soluble substrate, whereas the VS MN assay readout is the well area covered by a precipitating substrate.

**Table 2**
Sensitivity of MN assay results for variability in virus concentration.

| A/Tex/50/2012 (TCID50/well) | WHO MN assay[a] (20 h IM + serum) | | | | VS MN assay[d] (48h IM + CMC) | | |
|---|---|---|---|---|---|---|---|
| | Replicate 1 MN titer | Replicate 2 MN titer | GMT[b] | BT[c] | Replicate 1 MN titer | Replicate 2 MN titer | GMT |
| 10 | 64,000 | 32,000 | 45,255 | N | 32,000 | 16,000 | 22,627 |
| 30 | 32,000 | 32,000 | 32,000 | Y | 16,000 | 16,000 | 16,000 |
| 100 | 16,000 | 16,000 | 16,000 | Y | 16,000 | 32,000 | 22,627 |
| 300 | 8,000 | 8,000 | 8,000 | N | 8,000 | 16,000 | 11,341 |
| 450 | 8,000 | 8,000 | 8,000 | N | 8,000 | 16,000 | 11,341 |
| 600 | 8,000 | 8,000 | 8,000 | N | 8,000 | 8,000 | 8,000 |

[a] Cells were added to virus/serum mixtures in infection medium (IM) and incubated for 20 h prior to fixation and NP-ELISA.
[b] GMT: geometric mean titer.
[c] BT: Back-titration result in accordance with acceptation criteria: Y = yes, N = No.
[d] Virus/serum mixtures were added to cells for 90 min and replaced with IM + 1.6% carboxymethyl cellulose (CMC) for two days prior to fixation and ViroSpot (VS) immunostaining.

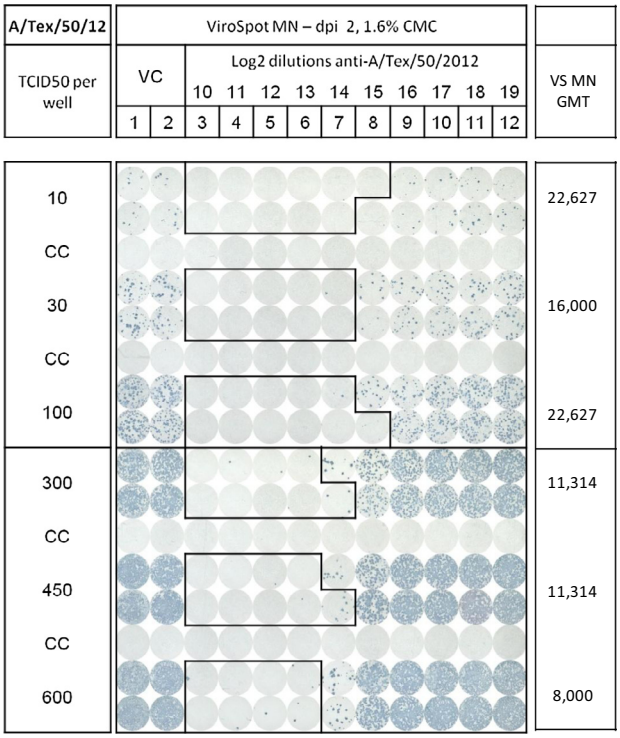


**Fig. 2.** ViroSpot MN results for six concentrations of A/Texas/50/2012 and ten two-fold dilutions of sheep immune serum. Boxed: ⩾90% inhibition of virus control (VC) value in columns 1 and 2. GMT: geometric mean titer of duplicates for each virus concentration. CC: cell control; VS MN: ViroSpot microneutralization; dpi: days post inoculation.

### 3.4. Antigenic characterization of epidemic strains

We further evaluated the ability of the ViroSpot MN assay to be used for the antigenic characterization of epidemic A(H3N2) influenza viruses, compared to the HI assay. To this end, virus stocks of seven A (H3N2) influenza viruses isolated between 2013 and 2015 in the Netherlands were produced and titrated. The antibody titers of post-infection ferret antisera raised against vaccine Ref. strains A/Victoria/361/2011, A/Texas/50/2012 and A/Switzerland/9715293/2013, and three of the epidemic strains, were determined against the homologous and heterologous strains (Table 4). Most of these viruses also displayed HA activity with guinea pig erythrocytes in the presence of oseltamivir. As shown in Table 4, in general, the antibody recognition profiles were similar in the Virospot MN assay and the HI assay, and corresponded to the antigenic clades the respective viruses belonged to. For example, antisera to influenza viruses A/Victoria/361/2011 and A/Texas/50/2012, both clade 3C.1 viruses, displayed relatively low titers to clade 3C.3 and 3C.2a viruses. The antiserum directed to virus A/Victoria/361/2011 displayed a higher degree of cross-reactivity, which was also observed by others [8]. Conversely, antiserum to clade 3C.3a virus A/Switzerland/9715293/2013 and clade 3C.2a virus A/Netherlands/1810/2015 displayed low titers to viruses from the other clades. The virus neutralization patterns were not caused by antibodies directed to NA, because antiserum directed to a reverse genetics H7N2 virus [14] failed to neutralize an A(H3N2) virus with an antigenically matching neuraminidase (data not shown). Furthermore, sera raised against an unrelated type of influenza virus (type B) did not display any non-specific neutralization of the A(H3N2) viruses.

## 4. Discussion

The emergence of influenza A(H3N2) viruses that fail to agglutinate erythrocytes in the absence or presence of oseltamivir in recent years, has complicated the antigenic characterization of these viruses with the traditionally used HI assay. This problem has been noted by us and others [8,9], and prompted the development and use of alternative assays for this purpose, like the virus neutralization assay.

In the late 90s, a MN assay was developed as an alternative for the HI assay for the detection of antibodies to avian influenza viruses of the H5N1 subtype in human serum samples [10]. However, this assay was deemed not suitable for the antigenic characterization of influenza viruses, because various influenza A(H3N2) viruses

**Table 3**
Antigenic typing of A(H3N2) reference viruses with post-infection ferret sera.

| Virus[a,b] | Back-titration | | WHO MN | | VS MN | | HI assay |
|---|---|---|---|---|---|---|---|
| | TCID50/well[b] | Serum | Titer[c] | Status[d] | Titer | Status | Match Status[e] |
| A/Wisconsin/67/2005 | 63 | X-187 | <320 | N | <320 | N | N |
| A/Uruguay/716/2007 | 93 | X-187 | <320 | N | <320 | N | N |
| A/Victoria/208/2009 | 102 | X-187 | 10,240 | M | 10,240 | M | M |
| **A/Victoria/210/2009 X-187** | **162** | **X-187** | **10,240** | **V** | **10,240** | **V** | **–** |
| A/Wisconsin/15/2009 | 141 | X-187 | <320 | N | 320 | N | N |
| A/Iowa/19/2010 | 139 | X-187 | 5120 | M | 2560 | M | M |
| A/Uruguay/716/2007 | 112 | IVR-165 | <40 | N | <40 | N | N* |
| A/Wisconsin/15/2009 | 177 | IVR-165 | 40 | N | 160 | N | N* |
| A/Iowa/19/2010 | 131 | IVR-165 | ? | ? | 5120 | M | M |
| A/Perth/10/2010 | 172 | IVR-165 | 40 | N | 160 | N | N* |
| **A/Victoria/361/2011 IVR165** | **293** | **IVR-165** | **2560** | **V** | **2560** | **V** | **–** |
| A/Hawaii/22/2012 | 66 | IVR-165 | 1280 | M | 1280 | M | M |
| A/Uruguay/716/2007 | 102 | X-223A | <40 | N | <40 | N | N* |
| A/Perth/16/2009 | 142 | X-223A | ? | ? | 320 | N | N |
| A/Hawaii/22/2012 | 94 | X-223A | 1280 | M | 2560 | M | M |
| **A/Texas/50/2012 X-223A** | **125** | **X-223A** | **2560** | **V** | **2560** | **V** | **–** |
| A/Hong Kong/146/2013 | 49 | X-223A | ? | ? | 2560 | M | M |
| A/South Africa/4655/2013 | 64 | X-223A | 160 | N | 160 | N | N |

[a] Boldface: vaccine strain.
[b] Back-titrations of virus test doses ranged from 49 to 293 TCID50, well within in the expected range of 20–500 TCID50.
[c] Sera were pre-diluted to obtain a dilution range extending from 32- to 64-fold lower through 2- to 4-fold higher than the homologous titer.
[d] V: Vaccine strain; M: match (⩽4-fold lower than homologous titer); N: non-match (>4-fold lower than homologous titer); ?: Unclear neutralization pattern due to low virus signal and high serum background.
[e] Based on hemagglutination inhibition (HI) documented in WHO annual consultation reports on the composition of influenza vaccines (www.crick.ac.uk/research/worldwide-influenza-Center);
* If no HI data were available, status was determined during preliminary ViroSpot MN assay testing.

**Table 4**
ViroSpot MN and HI-based antigenic typing of A(H3N2) epidemic virus isolates.

| Virus | Genetic clade | Passage history | HAU (-/+ OSEL)[a] | | | | A/Victoria/361/11 | | A/Texas/50/12 (X223A) | | A/Swiss/9715293/13 | | A/NL/2249/13 | | A/NL/1810/15 | | B/Phuket/3073/13[d] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | tRBC | | gRBC | | | | | | | | | | | | | |
| | | | - | + | - | + | VS-MN[b] | HI[c] | VS-MN | HI | VS-MN | HI | VS-MN | HI | VS-MN | HI | VS-MN | HI |
| A/Victoria/361/11 | 3C.1 | Mdck2 Siat2 Mdck4 | 64 | 0 | 64 | 8 | **320** | **480** | 80 | 640 | 160 | 320 | 1280 | 480 | 40 | 320 | <20 | <10 |
| A/Texas/50/2012 (X-223A) | 3C.1 | E9Mdck1 | 32 | 6 | 32 | 32 | 2560 | 1920 | **2560** | **2560** | 320 | 240 | 640 | 240 | 320 | 160 | <20 | <10 |
| A/Swiss/9715293/2013 | 3C.3a | E4E1Mdck1 | 64 | 6 | 32 | 32 | 2560 | 160 | 640 | 80 | **>10240** | **960** | 320 | 80 | 640 | 120 | <20 | <10 |
| A/NL/2249/2013 | 3C.3 | Mdck3 | 48 | 0 | 32 | 1 | 80 | nt | 80 | nt | 80 | nt | **160** | nt | 20 | nt | <20 | nt |
| A/NL/1810/2015 | 3C.2a | Mdck2 Siat2 Mdck4 | 8 | 6 | 16 | 8 | 80 | 60 | 40 | 240 | 40 | 120 | 80 | 80 | **>10,240** | **960** | <20 | <10 |
| A/NL/48/2015 | 3C.3 | Mdck1 | 8 | 0 | 16 | 0 | 320 | nt | 80 | nt | 40 | nt | 80 | nt | 40 | nt | <20 | nt |
| A/NL/573/2014 | 3C.3 | Mdck1 | 64 | 0 | 64 | 6 | 160 | 240 | 80 | 320 | 80 | 240 | 160 | 240 | 80 | 240 | <20 | <10 |
| A/NL/1679/2015 | 3C.2a | Mdck1 | 8 | 0 | 16 | 16 | 80 | 60 | 20 | 60 | 80 | 80 | 160 | 80 | 320 | 320 | <20 | <10 |
| A/NL/748/2014 | 3C.3 | Mdck2 | 8 | 0 | 16 | 16 | 80 | 640 | 40 | 640 | 20 | 320 | 160 | 480 | 40 | 320 | <20 | <10 |
| A/NL/1293/2015 | 3C.2a | Mdck3 | 8 | 8 | 16 | 16 | 640 | 120 | 80 | 320 | 20 | 320 | 40 | 160 | 2560 | 320 | <20 | <10 |

[a] Hemagglutination units (HAU) with or without oseltamivir (OSEL) for turkey red blood cells (tRBC) or guinea pig red blood cells (gRBC).
[b] Boldface: homologous titer.
[c] HI titers with gRBC + 20 nM OSEL; nt: not tested due to limiting HA activity.
[d] Homologous HI titer for B/Phuket/3073/13: 1920.

display different replication kinetics. As a result, plaque sizes may differ considerably, making the use of a classic plaque reduction assay difficult. Recently, a modification of a plaque reduction assay was described [8], in which cells are cultured under a solidified medium using Avicel overlay and quantification of the infected cell population (ICP). The amount of virus used in this assay is adjusted to give 20–85% infected cell area, which is also dependent on the variable replication kinetics of the different viruses tested. Thus, the amount of infectious units remains largely unknown and may vary between virus strains. To allow comparison of influenza virus antigenic properties in a reproducible manner, similarly to the HI assay, the amount of input virus ideally must be the same for each virus tested. To address this issue, the Virospot MN assay makes use of a standardized amount of input test viruses (100 TCID50) and an overlay of the cells with 1.6% carboxymethyl cellulose (CMC). CMC restricts plaque development even more so than Avicel [15], which is advantageous in terms of creating a better dynamic range for measuring inhibition of infectious units, even after a 2-day incubation period. This facilitates the testing of virus strains with the same protocol, independently of their replication kinetics, with relatively slow viruses able to produce sufficient signal to be detected, and at the same time preventing incidental non-neutralized units of rapidly replicating viruses to overgrow the entire well area. In general it makes sense to titrate virus preparations using the same readout as used in the virus neutralization assay. However, for the determination of infectious virus titers for the purpose of antigenic typing we consider an end-point dilution assay, i.e. a TCID50 assay, more appropriate, since it is more robust and objective than counting spots. Assessing infected and uninfected wells, as is done in the TCID50 assay, is independent of factors that may differ between virus isolates considerably, such as replication kinetics and spot sizes. Of note, the TCID50 assay was performed by incubating serial 5-fold virus dilutions for a period of time sufficient to guarantee scoring of all infected wells as positive by ViroSpot immunostaining, even those inoculated with a single infectious unit. Consequently, TCID50 values were independent of virus propagation kinetics and may reflect true infectious titers more accurately. Regardless, in general back-titration values showed good agreement with the expected inoculum concentration. An overview of the characteristics and differences of the WHO MN and ViroSpot MN assay is presented in Table 1. The ViroSpot MN assay offers several favorable properties: a standardized dose of input test virus with limited influence of the virus replication kinetics and cell-to-cell transmission, limiting its sensitivity to variation in input virus dose, in contrast to other MN formats, and infected cells are detected in an automated fashion, based on a precipitating substrate, suitable for high throughput use.

Compared to the HI assay, the ViroSpot MN assay is more complex and labor intensive to perform. One way to improve high throughput testing, could be the use of a number of set virus dilutions in duplicate without prior titration. Antigenic distance can subsequently be inferred by comparing neutralization titers between virus strains at similar virus concentrations as determined by back-titration. The use of MDCK-SIAT cell may also add to further improve the assay. However, even in its present form, the ViroSpot MN assay is suitable for the antigenic characterization of epidemic virus strains and its use may aid vaccine strain selection. The results obtained with the low passage isolates indicate that the assay can also be applied to field isolate testing. Future testing of additional isolates is required to further assess the sensitivity of the ViroSpot MN assay for this application.

Using A(H3N2) Ref. strains, which retained their capacity to agglutinate erythrocytes and which were characterized antigenically by HI assay, the ViroSpot MN assay was shown to correctly predict vaccine match status. The ViroSpot MN assay also proved suitable for the antigenic characterization of A(H3N2) epidemic strains isolated in the Netherlands during the 2014/2015 influenza season. The majority of A(H3N2) influenza viruses isolated during recent influenza seasons fail to agglutinate erythrocytes in the presence of oseltamivir and therefore antigenic characterization of these viruses solely relies on alternative (MN) assays. Antisera raised against vaccine strain A/Texas/50/2012 displayed poor VN antibody titers against such recent strains, which belong to genetic clades 3C.3 and 3C.2a. Antiserum raised against a 3C.2a virus A/Netherlands/1810/15 had high homologous titers, but failed to neutralize viruses of the other clades efficiently, confirming that these viruses were antigenically different. Of interest, the antigenically distinct clade 3C.2a viruses have become the dominant A(H3N2) viruses globally [16], which prompted the WHO to recommend a virus of this clade as the 2016 vaccine strain for the southern hemisphere, A/Hong Kong/4801/2014 [9]. The availability of a robust and reproducible MN assay is essential for the antigenic characterization of recent A(H3N2) influenza viruses. The ViroSpot MN assay offers attractive advantages over the WHO MN assay, including the relative insensitivity to variation in amount of infectious virus used in the test, independence from virus replication kinetics, automated virospot image capture and suitability for high throughput analyses. Of note, an infected cell population (ICP) MN assay has recently been described [8] and it would be of interest to compare the performance of the ViroSpot MN assay with this ICP

MN assay, which would require a multi-center effort using the same virus batches and serum samples. The availability of alternative VN assays may help the timely antigenic characterization of seasonal influenza viruses and the rapid recommendations of vaccine strains to be used in the subsequent influenza season, considered a priority by the WHO [17]. (http://www.who.int/influenza/vaccines/virus/4thmtg_improve_vaccine_virus_selection/en/).

## Conflict of interest

CvB, RJ, GP and BvG are employees of Viroclinics Biosciences BV. GR is consultant of Viroclinics Biosciences BV. The other authors declare no conflicts of interest.

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