SHORT COMMUNICATION

Replication kinetics of novel swine influenza A viruses: an approach to vaccine production

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ABSTRACT. Novel swine Influenza A viruses (IAVs) have been described in South America. The objective of this study was to evaluate the replication kinetics of novel swine IAVs as a first step in vaccine production. Different swine IAV lineages (H1N1, H1N2, and H3N2), infection doses (MOI: 1, 0.1, 0.01, 0.001, 0.0001, and 0.00001), harvest times (every 12 h), and substrates (MDCK and Vero cells) were used. For all IAV strains, MDCK cells were the most efficient substrate, generating titers of ≥128 HAU/50 μL with an MOI of 0.00001 at 60 h post-infection. These data may be useful in vaccine-producing laboratories.

Keywords: Influenza A virus, MDCK cells, Vero cells, antigen, vaccine, swine.

INTRODUCTION

Novel reassortant H1N2 and H3N2 swine influenza A virus (IAV) strains have recently been identified in commercial farms in Chile. These IAV strains are genetically and antigenically divergent from other IAVs described worldwide and circulate endemically in Chilean swine farms (Tapia *et al*., 2018, 2020). The hemagglutinin (HA) segments of these Chilean IAVs were likely introduced into swine from humans in the late 1980s and the early 1990s (Nelson *et al*., 2015). Other South American countries also have human-origin IAV lineages that circulate endemically in swine (Cappuccio *et al*., 2011; Resende *et al*., 2017). Commercial vaccines based on North American or European IAV strains would not be effective against these IAV strains, reinforcing the need to develop swine IAV vaccines with strains that represent antigenic clusters circulating at the local geographical level (Tapia *et al*., 2020). However, some laboratories do not have the expertise to produce swine IAV vaccines efficiently.

Vaccination is the primary method for preventing and controlling influenza in pigs. Most commercial swine IAV vaccines are based on whole-inactivated viruses, in which the major antigen is HA, a surface glycoprotein (Anderson *et al*., 2016). These vaccines are produced in embryonated chicken eggs or in cell cultures. Embryonated chicken eggs have been widely used in the production of IAV vaccines worldwide for over 70 years (CDC, 2022); however, this substrate has several drawbacks. Dependence on egg supply is a cause for concern, especially during high demand (McLean *et al*., 2016). This requires the availability of a large number of specific-pathogen-free eggs simultaneously and some-times within a short period of time, which can be scarce or unavailable in some countries. In addition, in resource-limited conditions, using embryonated eggs is labor intensive and requires considerable planning and effort to obtain sufficient eggs for inoculation (Hegde, 2015). Moreover, some IAV strains do not grow well in embryonated chicken eggs, such as human-origin H3N2 strains (CDC, 2019). Importantly, during growth and adaptation to embryonated chicken eggs, through serial passages, IAV strains are likely to acquire mutations that might change their antigenic properties, including glycosylation patterns, which could have an impact on antigenicity and decrease the efficacy of the vaccines produced (Skowronski *et al*., 2014; Zost *et al*., 2017).

Therefore, different mammalian cell lines have been evaluated for the production of whole-inactivated IAV vaccines in the last decade. The cell-based production of influenza vaccines has several advantages. Cell lines can be extensively stored for future production, avoiding dependence on egg supply. The process is more standardized and controlled; therefore, scalability is better with cell culture than with egg-based production systems. In general, mammalian influenza viruses grow well in mammalian cell lines, avoiding the time required for passage and adapting the viruses to embryonated chicken eggs. This also results in a decreased risk of generating mutations during viral passage and allows the maintenance of the antigenic characteristics of these IAVs in cell cultures as compared to embryonated chicken eggs (Manini *et al*., 2017; CDC, 2019; Tree *et al*., 2001). The main mammalian cell lines evaluated for the production of IAV vaccines were Madin-Darby Canine Kidney (MDCK), African green monkey kidney (Vero), Per.C6®, and AGE1.

CR® (Feng *et al.*, 2011; Manini *et al.*, 2017; CDC, 2019). Of these, MDCK and Vero cells have been well-studied and licensed for influenza vaccine production (Donis *et al.*, 2014; Genzel *et al.*, 2010).

The objective of this study was to evaluate the replication kinetics of novel swine IAVs in MDCK and Vero cells and to determine the optimal cell line, initial infectious dose, and harvest time to obtain high antigen (HA) titers. Using this approach, we aimed to establish an efficient protocol for obtaining stable antigen-specific IAV seed strains for use as vaccines against these viruses. In addition to an endemic pandemic H1N1 2009-like (A(H1N1)pdm09-like) swine IAV strain, we used novel reassortant H1N2 and H3N2 swine IAV strains previously identified in commercial farms in Chile.

MATERIALS AND METHODS

We used strains A/swine/Chile/VN1401-274/2014(H1N2), A/swine/Chile/VN1401-4/2014(H1N2), A/swine/Valparaiso/VN1401-559/2014(H1N1), and A/swine/Maule/VN1401-1824/2015(H3N2). They have been previously sequenced and deposited in GenBank (MF099149.1, MF099073.1, MK160103.1, MF099352.1) and genetically characterized. The strains represent four different Chilean swine IAV lineages, as previously described (Tapia *et al.*, 2018, 2020). According to the H1 classification described by Anderson *et al.* (2016), the strains A/swine/Chile/VN1401-274/2014(H1N2) and A/swine/Chile/VN1401-4/2014(H1N2) were classified within the clade Other-Human-1B.2, whereas the strain A/swine/Valparaiso/VN1401-559/2014(H1N1) is an A(H1N1)pdm09-like strain classified within clade 1A.3.3.2. The strains A/swine/Chile/VN1401-274/2014 (H1N2), A/swine/Chile/VN1401-4/2014 (H1N2), and A/Maule/Chile/VN1401-1824/2015(H3N2) were genetically distant from other IAVs identified in swine and humans globally (Tapia *et al.*, 2018, 2020).

All procedures were approved by the Biosafety Institutional Committee (Certificate Number 104-07-11-2017) and the Institutional Committee for Animal Care and Use (CICUA) of the University of Chile (Certificate Number 02-2016). The study was conducted in accordance with local legislation and institutional requirements. Viral isolation and propagation of viruses were performed under BSL-2 conditions according to international recommendations (Meechan & Potts, 2020).

First, these strains were titrated using a plaque assay, which is an accurate method for the direct quantification of infectious virions by counting discrete plaques (infectious units and cellular dead zones) in cell culture (Baer & Kehn-Hall, 2014). For this assay, 6-well plates were seeded with 600,000 MDCK cells per well, using 3 mL of minimum essential medium (MEM) supplemented with 10% fetal bovine serum and 1% antibiotic–antimycotic solution, and incubated for 24 h at 37 °C and 5% CO_2. The following day, confluent MDCK cells were washed twice with 1x phosphate-buffered saline (PBS) and inoculated with 200 µL of ten-fold serial dilutions of each IAV strain (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4},

10^{-5} and 10^{-6}). The plates were incubated for 1 h at 37 °C and 5% CO_2 to allow for virus absorption. Then, the inoculum was removed, 2 mL of solid IAV growth medium (MEM, 1% dextran, 5% $NaHCO_3$, 0.3% bovine serum albumin, 2% purified Oxoid™ agar (Basingstoke, Hampshire, UK), and 1 µg/mL trypsin treated with N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK)) was added to each well, and the plates were incubated for 48 h at 37 °C and 5% CO_2. Next, 1 mL of 4% formaldehyde was added to each well, and the plates were incubated for 1 h at RT. The solid IAV growth medium with formaldehyde was removed and 0.5% crystal violet was added to visualize the viral plaques in the MDCK monolayers. The plaques were counted, and the virus titer was expressed in plaque-forming units per mL (PFU/mL). Each IAV strain was titrated in triplicates.

Once the titer of each swine IAV strain was determined, 6-well plates were seeded with MDCK and Vero cells (600,000 cells per well) using 3 mL of MEM supplemented with 10% fetal bovine serum and 1% antibiotic–antimycotic solution, and incubated for 24 h at 37 °C and 5% CO_2. The MDCK and Vero cell lines were kindly provided by Dr. Sagar Goyal (University of Minnesota, MN, USA). Confluent cells were washed twice with PBS and inoculated with 200 µL of ten-fold serial dilutions of each strain, corresponding to a multiplicity of infection (MOI) of 1, 0.1, 0.01, 0.001, 0.0001, and 0.00001 (MOI refers to the number of virions added per cell during infection, i.e., a MOI of 1 means 1 virion for every cell, while a MOI of 0.00001 means 1 virion for every 100,000 cells). After incubation for 1 h at 37 °C and 5% CO_2, the monolayers were washed twice with PBS, and 3 mL of IAV growth medium (MEM supplemented with 1 µg/mL TPCK-treated trypsin, 0.3% bovine serum albumin, and 1% antibiotic–antimycotic solution) was added. The plates were incubated at 37 °C and 5% CO_2. The monolayers were observed for cytopathic effect (CPE), and 50 µL of supernatant was collected every 12 h post-infection to determine the HA titer using a standard hemagglutination assay (Kitikoon *et al.*, 2014). The HA titer was expressed as hemagglutination units per 50 µL (HAU/50 µL) and measured until the CPE exceeded 75% of the monolayer. The most efficient type of cell line, MOI, and harvest time were used to reproduce this assay on a larger scale, using roller bottles with an area of 1,700 cm2 (Corning®, NY, USA). The assay was performed in triplicate.

The MOI of 0.00001 generated the highest HA titer for all IAV strains inoculated in MDCK cells, reaching a peak of 128 HAU/50 µL at 48 h post-infection for the strains A/swine/Chile/VN1401-274/2014(H1N2), and 256 HAU/50 µL at 60 h post-infection for the strains A/swine/Chile/VN1401-4/2014(H1N2), A/Valparaiso/Chile/VN1401-559/2014(H1N1), and A/swine/Maule/VN1401-1824/2015(H3N2) (Figure 1).

In Vero cells, the experiment was extended up to 120 h post-infection due to the slow development of the CPE, at which time the strains reached the maximum HA titer. The strain A/swine/Chile/VN1401-274/2014(H1N2) reached a titer of 32 HAU/50 µL with the MOI 0.01 and 0.001, whereas the
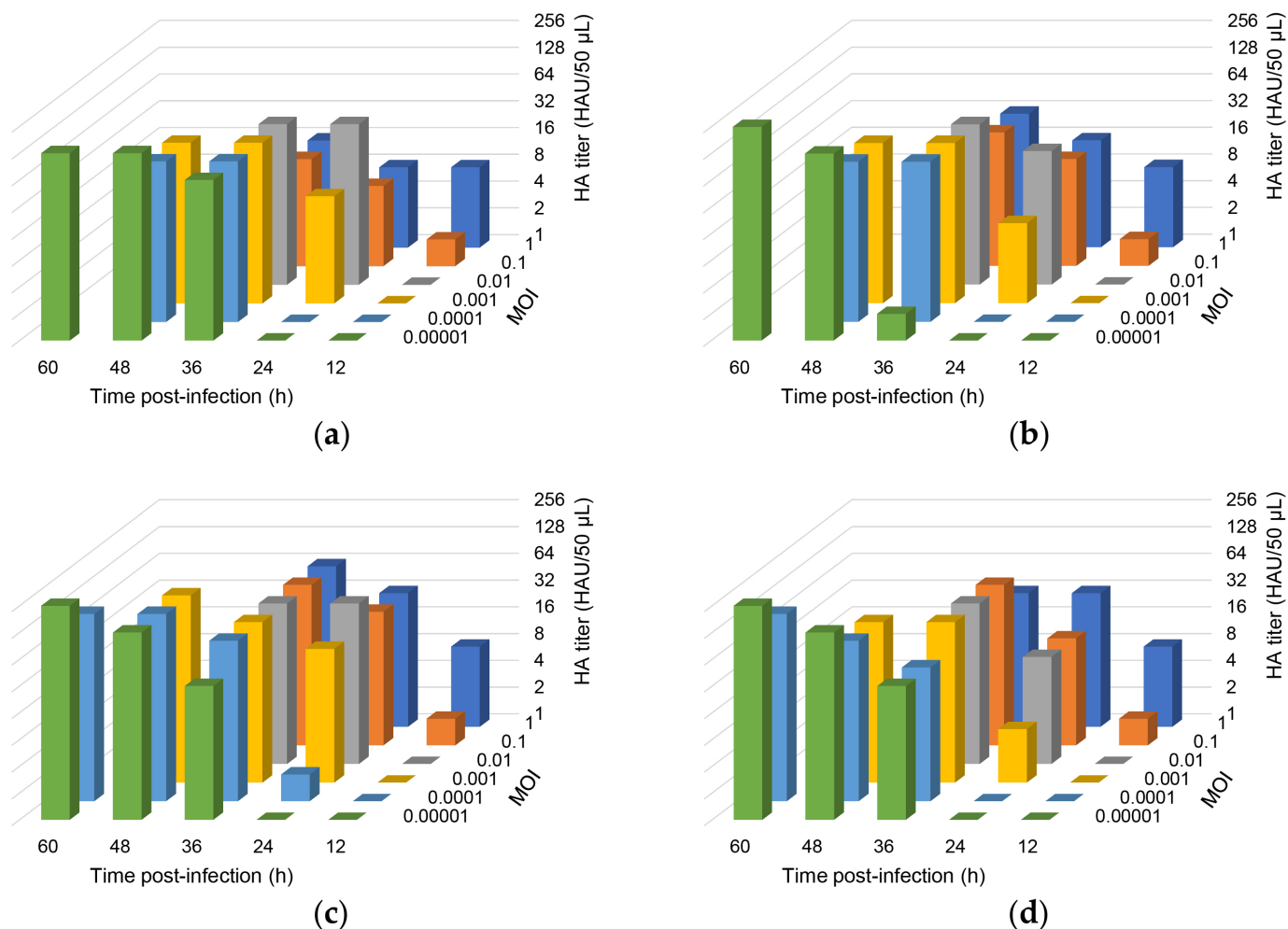
Figure 1.
Replication of swine IAV strains in MDCK cells. MDCK cells were inoculated with strains A/swine/Chile/VN1401-274/2014(H1N2) (a), A/swine/Chile/VN1401-4/2014(H1N2) (b), A/swine/Valparaiso/VN1401-559/2014(H1N1) (c), and A/swine/Maule/VN1401-1824/2015(H3N2) (d). MOI of 1, 0.1, 0.01, 0.001, 0.0001 and 0.00001, were used for each strain. HA titers were calculated at 12, 24, 36, 48, and 60 h post-infection.

strains A/swine/Chile/VN1401-4/2014(H1N2) and A/swine/Maule/VN1401-1824/2015(H3N2) reached a titer of 16 and 64 HAU/50 µL, respectively, with the MOI 0.1. Strain A/Valparaiso/Chile/VN1401-559/2014(H1N1) did not generate CPE or HA titers (Figure 2).

RESULTS AND DISCUSSION

The MDCK cell line and the MOI of 0.00001 were the most efficient in replicating IAV strains. This infectious dose was then selected to reproduce the assay on a larger scale in roller bottles to confirm the results obtained in the plates. Harvesting was carried out 60 h post-infection. A harvest volume of 300 mL per bottle was obtained for each strain, with a titer of 256 HAU/50 µL for all IAV strains.

In this study, we evaluated the replication kinetics of novel reassortant swine IAV strains and determined the optimal cell line, initial infectious dose, and harvest time for obtaining high HA titers. These strains belong to different clades and subtypes, representing the genetic and antigenic diversity of the swine IAVs circulating in Chile (Tapia *et al.*, 2018, 2020).

All IAV strains replicated efficiently in MDCK cells, generating equivalent infection dynamics and HA titers. High HA titers can be achieved using low MOIs. The highest HA titers were obtained at the lowest initial infectious dose (MOI = 0.00001). This inverse relationship between the MOI and virus yield, determined by the HA assay, has been previously described (Isken *et al.*, 2012; Petiot *et al.*, 2018; Rimmelzwaan *et al.*, 1998). Low HA titers with high MOIs could be due
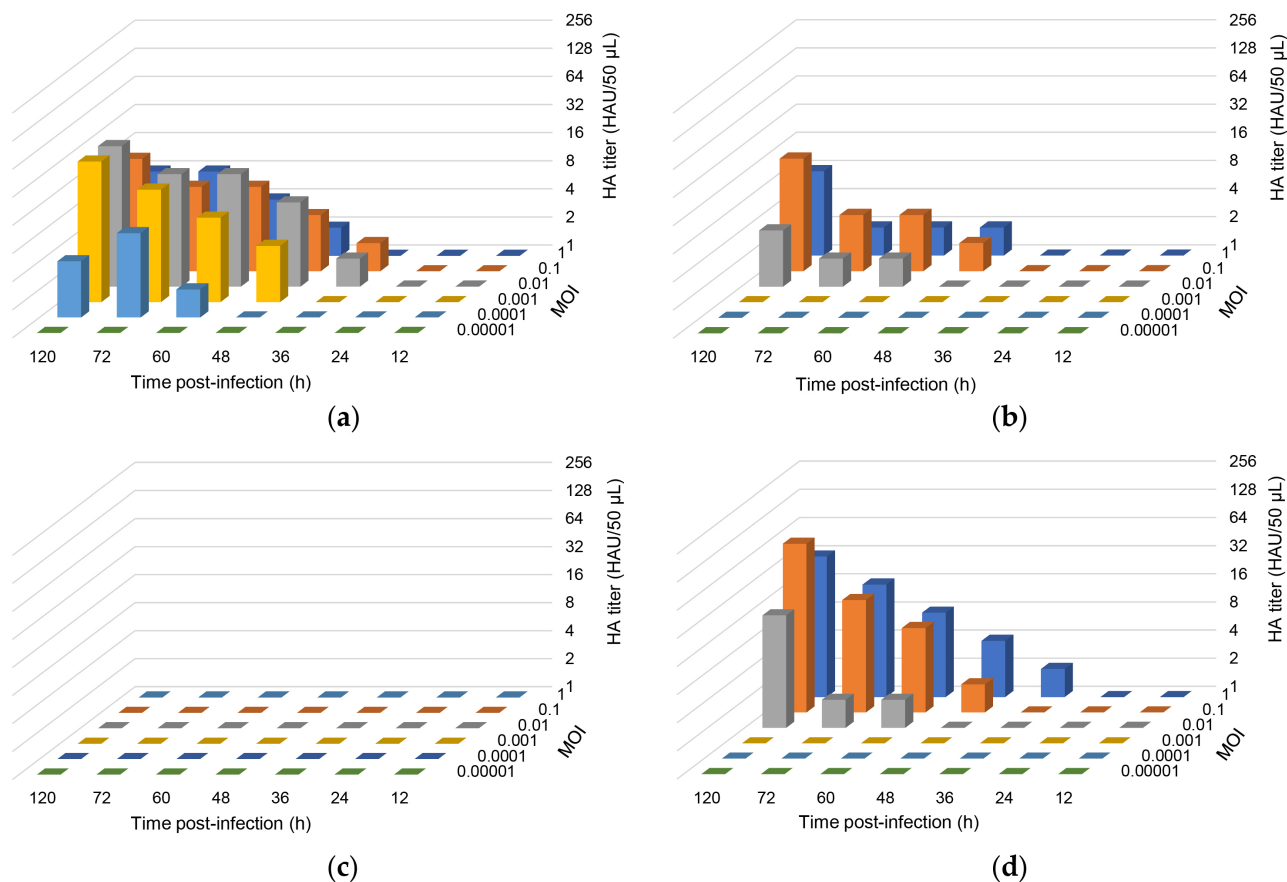
Figure 2.
Replication of swine IAV strains in Vero cells. Vero cells were inoculated with strains A/swine/Chile/VN1401-274/2014(H1N2) (a), A/swine/Chile/VN1401-4/2014(H1N2) (b), A/swine/Valparaiso/VN1401-559/2014(H1N1) (c), or A/swine/Maule/VN1401-1824/2015(H3N2) (d). MOI of 1, 0.1, 0.01, 0.001, 0.0001 and 0.00001, were used for each strain. HA titers were calculated at 12, 24, 36, 48, 60, 72, and 120 h post-infection.

to the presence of non-infectious biologically active influenza virus particles (niBAPs) or other types of interfering particles that lack replication capacity, specifically non-infectious cell-killing particles (niCKPs) (Brooke, 2014). These non-infectious particles have the potential to influence the course of pathogenesis through their capacity to stimulate or suppress antiviral responses, and in the case of niCKPs, to drive cells into apoptosis (Marcus *et al.*, 2009). These swine IAV strains are likely to generate a high proportion of niCKPs that are sufficiently diluted at low MOIs but play an important role in inducing apoptosis at higher MOIs (Isken *et al.*, 2012). Specifically, this could be the case for strain A/swine/Chile/VN1401-274/2014(H1N2), which reached a lower HA titer (1 log base 2) than the other strains. Possibly, higher HA titers (>256 HAU/50 µL) would have been reached if we used lower MOIs. The cells would have remained viable for a longer period (>60 h), allowing a higher HA titer to be achieved. Isken *et al.* (2012) showed that strain-specific differences in HA titers and the induction of apoptosis are

less pronounced with a reduction in the MOI, which is beneficial for robustness in vaccine production processes, as process conditions and harvest time can be kept constant regardless of the strain used (Isken *et al.*, 2012).

In general, IAV strains reached the maximum HA titer at 60 h post-infection in MDCK cells at an MOI of 0.00001. Therefore, this was the optimal harvest time for this MOI. Knowing the optimal harvest time for a given MOI is very useful in vaccine production, as the production of each batch can be better planned and scheduled. It must be noted that, on a large scale, it is not possible to visualize CPE to determine harvest time.

In contrast to MDCK cells, lower HA titers were obtained in Vero cells, indicating less efficient replication kinetics. No CPE or HA titers were obtained with the MOI of 0.00001 at any time post-infection. In fact, the pandemic-like strain A/Valparaiso/Chile/VN1401-559/2014(H1N1) could not replicate in Vero cells. Some IAV strains have been reported to be unable to generate high viral load titers in Vero cells (Liu

et al., 2009). This may be because Vero cells rapidly inactivate exogenous trypsin, which restricts the replication of influenza viruses (Kaverin & Webster, 1995). Trypsin has a negative effect on interferon (IFN)-induced antiviral proteins (Seitz *et al.*, 2012) and cleaves HA to induce cellular infection (Klenk *et al.*, 1975). In addition, canine IFN-induced myxovirus resistance protein 1 (Mx1) produced by MDCK cells cannot inhibit IAV replication (Seitz *et al.*, 2010), making it advantageous over other cell lines used in the replication of this virus (Hegde, 2015; Manini *et al.*, 2017).

In conclusion, we evaluated the optimal parameters for growth of novel swine IAV strains recently described in Chile. The optimal cell line, initial infectious dose, and harvest time were determined. These factors are critical for vaccine production, particularly for whole-inactivated vaccines. Although the optimal MOI depends on the virus strain, in general, this protocol could be useful for laboratories producing swine IAV vaccines that are just beginning or have less experience. This is important because other South American countries also have human-origin swine IAVs that are genetically and antigenically different from commercial vaccine strains (Cappuccio *et al.*, 2011; Resende *et al.*, 2017) and might have to produce their own vaccines for effective prevention and control.

DECLARATIONS

Competing interests statement

Ethics statement

Not applicable.

Availability of data and materials

The dataset supporting the conclusions of this study is available in the Supplementary Data.

Authors' contributions

Conceptualization, RT and VN; methodology, RT and RAM; validation, RT and VN; formal analysis, RT; investigation, RT; resources, RT, RAM and VN; data curation, RT; writing—original draft preparation, RT; writing—review and editing, RT, RAM and VN; visualization, RT; supervision, RT and VN; project administration, RT; funding acquisition, RT, RAM, and VN. All authors have read the final version of the manuscript and accept responsibility for the veracity and originality of this work.

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