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Stability of retroviral pseudotypes carrying the hemagglutinin of avian influenza viruses under various storage conditions

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ABSTRACT

Retroviral pseudotypes are broadly used as safe instruments to mimic the structure and surface of highly pathogenic viruses. They have been employed for the discovery of new drugs, as diagnostic tools in vaccine studies, and part of serological assays. Because of their widespread use in research and their potential as tools for quality control, it is important to know their shelf life, stability, and best storage conditions. In this study, we produced pseudotypes carrying the *lacZ* reporter gene and the hemagglutinin (HA) of avian influenza virus subtypes H5 and H7 to investigate their stability under various storage conditions. We produced pseudotypes with titers of approximately 10^6 RLU/mL, which decreased to 10^5–10^6 RLU/mL after short-term storage at 4 °C (up to 4 weeks). Stability was maintained after long-term storage at −20 °C (up to 12 months), even under storage variations such as freeze-thaw cycles. We conclude that, although the titers decreased by 1 log_{10} under the different storage conditions, the remaining titers can be readily applicable in many techniques, such as neutralization assays. These findings show that large quantities of retroviral pseudotypes can be safely stored for short- or long-term use, allowing standardization and reduced variation in assays involving retroviral pseudotypes.

1. Introduction

Retroviral pseudotypes are non-replicative chimeric viral particles carrying surface proteins-of-interest, and a unique gene as genetic material, for example, a reporter gene; thus, the biosafety requirements for studying a pathogen are reduced (García and Lai, 2011). In addition, they represent a versatile platform because particles with different surface proteins from many viruses can be generated, resulting in different characteristics, biological activity, and stability (Sawoo et al., 2014). Retroviral pseudotypes carrying the hemagglutinin from influenza viruses (HA pseudotypes) are used as safe surrogates to mimic the structure and surface of highly pathogenic viruses such as avian and human influenza virus, in order to investigate the biological functions mediated by the envelope proteins derived from these viruses, for drug and vaccine development, and in diagnosis (García and Lai, 2011; Sawoo et al., 2014; Wang et al., 2014; Joglekar and Sandoval, 2017; Ferrara and Temperton, 2017; Xiao et al., 2018).

The increased number of outbreaks of Low-Pathogenicity Avian Influenza (LPAI) and High-Pathogenicity Avian Influenza (HPAI) by H5 and H7 viruses produce high mortality in poultry, causing devastating losses in poultry production worldwide; this is relevant for public and animal health and has a significant economic impact (Molesti et al., 2014; Nao et al., 2017). Avian influenza A virus subtypes H5 and H7 are responsible for numerous outbreaks in poultry (Belser et al., 2009, 2013) and have been responsible for numerous zoonotic transmissions from poultry to humans (Poovorawan et al., 2013). H5N1 HPAI viruses, first reported in Hong Kong in 1997, have been circulating in poultry for nearly two decades. In addition to infection of avian species, it has been reported that H5N1 HPAI viruses are occasionally transmitted to humans and cause severe pneumonia with high case-fatality rates (Lu et al., 2014). In China as of May 2015, 657 human cases of influenza H7N9 were confirmed (De Jonge et al., 2016). HPAI outbreaks by H7N3 have occurred three times in the Americas in the past 10 years and caused severe economic losses in the affected regions (Lu et al., 2014). In Mexico, the avian influenza virus was identified for the first time in 1994 and corresponded to the low-pathogenicity H5N2 subtype. Due to the lack of proper diagnosis of what was considered an exotic disease at the time, the infection spread to the main poultry areas of the country

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(Horimoto et al. 1995). In June 2012, H7N3 HPAI outbreaks occurred in poultry on commercial farms in Mexico, affecting broilers, breeders, layers, and backyard poultry in the Mexican states of Jalisco, Aguascalientes, Guanajuato, and Puebla, regions with high-poultry density. In that outbreak, 4.9 million birds were slaughtered and destroyed, out of an estimated population of 9.3 million in the quarantined area (López-Martínez et al., 2013), and two cases of human infection following exposure to infected poultry were confirmed, both cases presenting conjunctivitis (Afanador-Villamizar et al., 2017; López-Martínez et al., 2013; Spackman et al. 2014, Vázquez-Mendoza et al., 2014).

The use of HA-pseudotypes has been shown to be highly efficient for the detection of neutralizing antibodies, rendering them ideal for the study of cross-protective responses against multiple subtypes of influenza virus with pandemic potential (Alberini et al., 2009; Du et al., 2010; Molesti et al., 2014; Wang et al., 2008, 2010). With this aim, if the pseudotypes are mixed with serum from an individual with specific antibodies directed against the HA, along with neutralizing antibodies, transference of the reporter gene will be blocked because the pseudotypes will not interact with the receptors in the cells, but instead with the neutralizing antibodies. Therefore, the application of HA-pseudotypes as analytical tools guarantees the evaluation of their quality control and stability. In this regard, it has been demonstrated that stability can be influenced by various factors, including storage temperature and freeze-thaw frequency (Higashikawa and Chang, 2001; Wang et al., 2010). In this study, in order to investigate their stability under various storage conditions and after several freeze-thaw cycles, HA pseudotypes of H5 and H7 subtypes of avian influenza virus were produced. We found that the titer of the pseudotypes decreased by 1 log unit under the different temperatures of storage and after freeze-thaw cycles, although the remaining HA-pseudotype titers remained optimal and applicable in neutralization assays.

2. Materials and methods

2.1. Cell culture

For production of pseudotypes and for titration of pseudotype batches, HEK 293T cells (ATCC: CRL-11268TM; Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 5% glucose, 1% L-glutamine, 1% nonessential amino acids, 1% HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and supplemented with 10% Fetal Calf Serum (FCS) (Wang et al., 2008). In case of the titration assays, 10,000 International Units (IU)/mL penicillin and 10,000 μg/mL streptomycin were added to the medium.

2.2. Plasmids

Lentiviral gag-pol, rev plasmids, and *lacZ* reporter (pCgpV, pRSV-Rev, and pSMPUW-MNDnLacZ, respectively) belong to the ViraSafe™ Lentiviral Bicistronic Expression System kit (Cell Biolabs, Inc., San Diego, CA, USA). Plasmids encoding *Human Airway Trypsin-like protease* (HAT) (pHAT), Neuraminidase (NA) of influenza A (H1N1) pandemic 2009 isolated A/California/04/2009 (H1N1) (pCA NA), and M2 protein A/PR/8/34 (pPR M2) were kindly provided by Carol D. Weiss (U.S: Food and Drug Administration [FDA], Bethesda, MD, USA). The M2 protein has been reported by some authors to be required for HA protection during its biogenesis in the cell (García and Lai, 2011; Alvarado-Facundo et al., 2015). The HA consensus sequence of H5 was codon-optimized for expression in HEK 293T cells and chemically synthesized (T4 OLIGO, Guanajuato, Mexico). The gene was cloned into the pVAX-1 expression plasmid (pHAH5) and confirmed by sequencing. HA genes A/cinnamonteal/Mexico/2817/2006(H7N3) (GenBank: AGG86912.1) and A/chicken/Jalisco/CPA1/2012/ (H7N3) (GenBank: AFN85519.1) were codon-optimized for expression in HEK 293T cells and chemically synthesized (T4 OLIGO). The genes were cloned into the pVAX-1 expression plasmid (pHAH7-06, pHAH7-12) and confirmed by sequencing.

2.3. Production of influenza HA pseudotypes

Influenza HA pseudotypes carrying a *lacZ* reporter gene were produced by HEK 293T cells co-transfected with the X-tremeGENE HP DNA Transfection Reagent (Roche) and the following plasmids: ViraSafe™ Lentiviral Bicistronic Expression System including pSMPUW-MNDnLacZ, pCgpV, and pRSV-Rev; pHAT, pPR M2, and pCA NA, and one of the following plasmids: pHAH7/06; pHAH7/12, or 2 pHAH5. At 18 h post-transfection, cells were fed fresh medium. Supernatants were collected 48 h post-transfection, filtered through a 0.45-mm low protein-binding filter and maintained at 4, −20, and −80 °C (Wang et al., 2008, 2010).

2.4. Titration of the pseudotype batches

HA-pseudotype activity was evaluated through the *lacZ* reporter gene activity in HEK 293T cells. Galacto-Star kit (Applied Biosystems) was used to detect LacZ activity. 48 h after contact of HA-pseudotypes with the cells on 96-well plates, the cells were lysed with lysis buffer, 100 μL of Galacto-Star substrate were added, incubated for 60 min, the signal of chemiluminescence was measured in a luminometer with one second of integration and expressed in Relative Luminescence Units per milliliter (RLU/mL). Data reported are expressed as the mean from testing in triplicate assays, the coefficient of variation was determined and control wells containing only cells were included to discard background noise (Carnell et al., 2015; Wright et al., 2009). A coefficient of variation of ≤20 was considered acceptable for this bioassay (FDA, 2016).

2.5. Stability testing

Titers of HA-pseudotypes maintained at 4 and −80 °C were titrated at 7, 14, 21, and 28 days for short-term evaluation (Wang et al., 2010), while HA pseudotypes maintained at −20 °C were titrated at 3, 5, 9, and 12 months for long-term evaluation by Molesti et al. (2014) and Wang et al. (2010).

2.6. Freeze-thaw cycles

HA-pseudotype aliquots were frozen at −80 °C and then thawed to room temperature for a total of five freeze-thaw cycles; after each freeze-thaw cycle, HA-pseudotype titers were measured by transfer of the *lacZ* reporter gene onto HEK 293T cells (see Section 2.4 Titration of the pseudotype batches) (Higashikawa and Chang, 2001; Wang et al., 2010).

3. Results

3.1. Production of HA-pseudotypes

We produced HA-pseudotypes (H5) carrying the consensus HA of avian influenza virus isolated in Mexico subtype H5, HA-pseudotypes (H7/06) carrying the HA of vaccine strain employed in Mexico in 2012, and HA-pseudotypes (H7/12) carrying the HA subtype H7 of an HPAI strain responsible for the outbreak in Mexico in 2012, all of these rendering titers of approximately 10^6 RLU/mL, thus corroborating efficient production (Fig. 1).

3.2. Stability of HA-pseudotypes at 4 °C

HA-pseudotypes were briefly stored at 4 °C for different periods up to 1 month; then the activity of the reporter gene on HEK 293T cells was measured. The initial titer of HA-pseudotypes (H5) was 1.5×10^6 RLU/mL, and it gradually decreased to 1.1×10^6 RLU/mL on day 28,

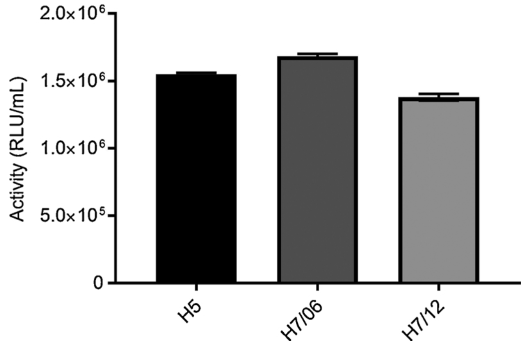

Fig. 1. Production of HA-pseudotypes. The titers were measured through the expression of the *lacZ* reporter gene on HEK 293T cells and activity was expressed in relative luminescence units per milliliter (RLU/mL). Data is expressed as mean ± standard deviation (SD) from three replicates and a coefficient of variation < 20 per test.

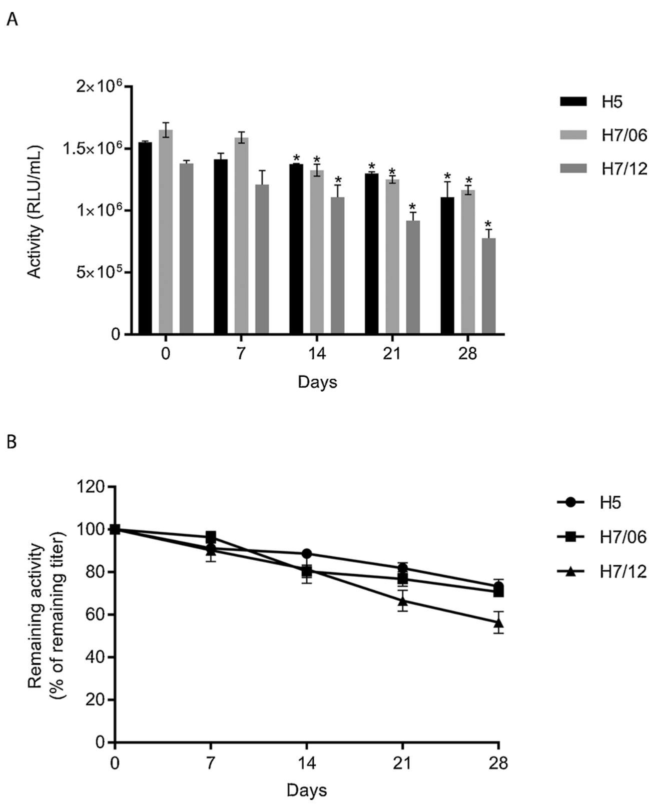

Fig. 2. HA-pseudotype storage stability at 4°C. HA-pseudotype titers were measured in HEK 293T cells and activity was expressed in relative luminescence units per milliliter (RLU/mL) (A). Comparison of remaining activity (B). Data is expressed as mean ± standard deviation (SD) from three replicates, a coefficient of variation < 20 per test and analyzed by one-way ANOVA (Analysis of variance) ($p < 0.01$*).

with 70% activity remaining. In the case of HA-pseudotypes (H7/06), the initial titer was 1.6×10^6 RLU/mL, and the titer gradually decreased to 1.16×10^6 RLU/mL on day 28, with 60% of activity remaining. The initial titer of HA-pseudotypes (H7/12) was 1.3×10^6 RLU/mL and this decreased to 7.6×10^5 RLU/mL on day 28, with 50% activity remaining. The titers of HA-pseudotypes demonstrated a statistically significant difference starting at day 14 (Fig. 2).

3.3. Stability of HA-pseudotypes at −80 °C

HA-pseudotypes were briefly stored at −80 °C for different periods up to 1 month, and the activity of the reporter gene on HEK 293T cells was measured. The initial titer of HA-pseudotypes (H5) was 1.5×10^6 RLU/mL, and the titer decreased to 1.4×10^6 URL/mL, with 90% activity remaining, exhibiting a statistically significant difference on day 28. The titer of HA-pseudotypes (H7/06) decreased from 1.6×10^6 to 1.4×10^6 URL/mL on day 28, with 80% activity remaining, demonstrating a statistically significant difference from day 14. The initial titer of HA-pseudotypes (H7/12) was 1.3×10^6 RLU/mL, it decreased to 1.2×10^6 URL/mL on day 28, with 80% activity remaining, showing a statistically significant difference from day 14 (Fig. 3).

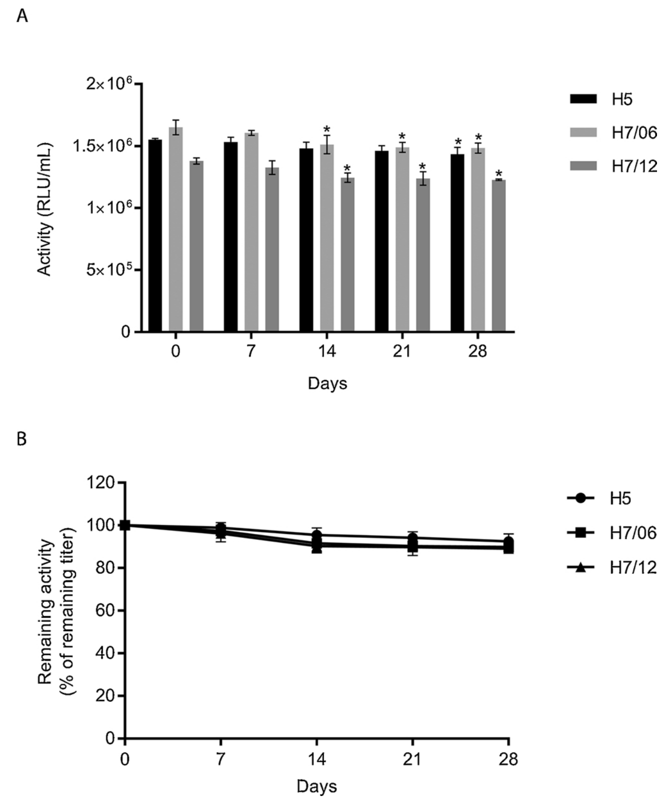

Fig. 3. HA-pseudotype storage stability at -80°C. HA-pseudotype titers were measured in HEK 293T cells and activity was expressed in relative luminescence units per milliliter (RLU/mL) (A). Comparison of remaining activity (B). Data is expressed as mean ± standard deviation (SD) from three replicates, a coefficient of variation < 20 per test and analyzed by one-way ANOVA (Analysis of variance) ($p < 0.01$*).

3.4. Stability of HA-pseudotypes at −20 °C

HA-pseudotypes were stored at −20 °C for different periods up to 1 year. Then, the activity of the reporter gene on HEK 293T cells was measured. The initial titer of HA-pseudotypes (H5) was 1.5×10^6 RLU/mL. After storage, the titer was 1×10^6 RLU/mL on month 12, with 65% of activity remaining, showing a statistically significant difference from month 3. Regarding HA-pseudotypes (H7/06), the initial titer was 1.6×10^6 RLU/mL. It decreased to 10^6 RLU/mL on month 9, and it decreased by one logarithm at month 12, with 49% remaining activity, demonstrating a statistically significant difference from month 5. In the case of HA-pseudotypes (H7/12), the initial titer was 1.3×10^6 RLU/mL; the latter it decreased to 1×10^6 RLU/mL on month 5, and it decreased by one logarithm at month 9, with 55% activity remaining, exhibiting a statistically significant difference from month 3 (Fig. 4).

3.5. Stability of HA-pseudotypes after freeze-thaw cycles

HA-pseudotypes were subjected to five freeze-thaw cycles; then, the

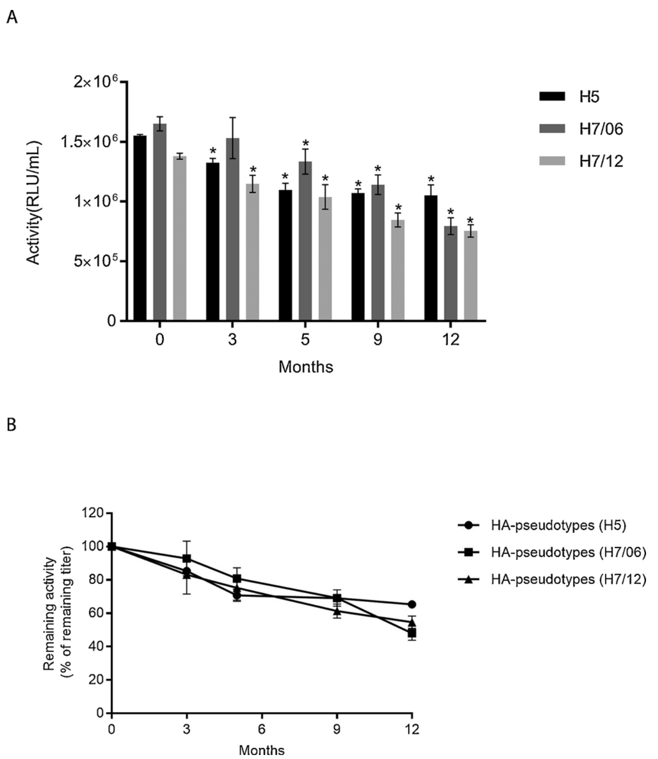

Fig. 4. HA-pseudotype storage stability at -20°C. HA-pseudotype titers were measured in HEK 293T cells and activity was expressed in relative luminescence units per milliliter (RLU/mL) (A). Comparison of remaining activity (B). Data is expressed as mean ± standard deviation (SD) from three replicates, a coefficient of variation < 20 per test and analyzed with one-way ANOVA (Analysis of variance) ($p < 0.01$*).

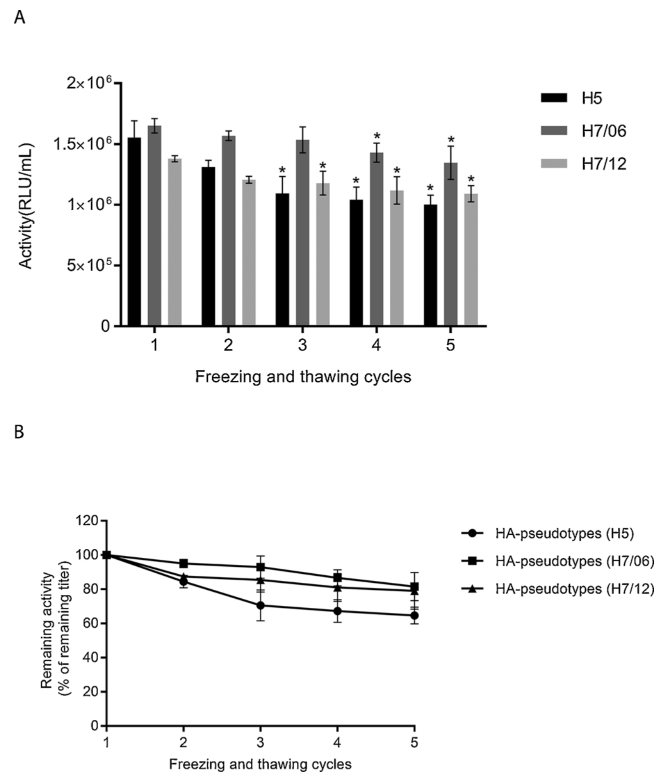

Fig. 5. The influence of freeze-thaw cycles. HA-pseudotype titers were measured in HEK 293T cells and activity was expressed in relative luminescence units per milliliter (RLU/mL) (A). Comparison of remaining activity (B). Data is expressed as mean ± standard deviation (SD) from three replicates, a coefficient of variation < 20 per test and analyzed with one-way ANOVA (Analysis of variance) ($p < 0.01$*).

activity of the reporter gene in HEK 293T cells was measured. The titer of HA-pseudotypes (H5) decreased from 1.5×10^6 to 1×10^6 RLU/mL after five cycles, with 65% remaining activity, revealing a statistically significant difference from the third cycle. In this respect, the titer of HA-pseudotypes (H7/06) decreased from 1.6×10^6 to 1×10^6 RLU/mL after five cycles, with 82% activity remaining, showing a statistically significant difference from the fourth cycle. HA-pseudotypes (H7/12) decreased from 1.3×10^6 to 1×10^6 RLU/mL after five cycles, with 79% remaining activity, exhibiting a statistically significant difference from the third cycle (Fig. 5).

4. Discussion

Avian influenza outbreaks in poultry have been increasing in frequency since 1997 (Capua and Alexander, 2004), and avian subtypes H5 and H7 have both caused influenza outbreaks in human populations (Poovorawan et al., 2013). From a veterinary viewpoint, serological and biological surveillance is necessary, not only as monitoring systems for avian influenza viruses circulating among poultry species but also as a prevention and control strategy for strains with pandemic potential (Naeem et al., 2003). The use of retroviral pseudotypes carrying the influenza hemagglutinin (HA) is considered a safe alternative that substitutes for the use of the wild influenza virus in many techniques such as neutralization assays, thus reducing the biological risks involved (García and Lai, 2011; Carnell et al., 2015; Wallerstrom et al., 2014; Wang et al., 2014). It is important to know their stability, shelf life, and best storage conditions; several studies suggest -80 °C as the adequate temperature for storing batches of pseudotypes during production (Alvarado-Facundo et al., 2015; Sawoo et al., 2014; Zimmermann et al., 2011), while some researchers have investigated other storage conditions, and their pseudotype stocks were highly stable at basic cold-chain storage conditions of −20 °C for over 6 months and +4 °C for 2–4 weeks (Molesti et al., 2014; Wang et al., 2010 and Wright et al., 2009). In this study, we investigated HA-pseudotype stability, because it can be influenced by various factors including temperature and freeze-thaw frequency during transportation and storage (Higashikawa and Chang, 2001). We further investigated best storage conditions for the short and long-term suggested by Molesti et al. (2014) and Wang et al. (2010), in order to understand HA-pseudotype stability at basic cold-chain storage conditions of 4 °C and −20 °C. Activity was evaluated through the determination of the titers of the HA-pseudotypes by transferring the *lacZ* reporter gene in HEK 293T cells because *lacZ* has been the reporter gene in studies involving these variations (Molesti et al., 2014). We successfully produced pseudotypes carrying the HA of subtypes H5 or H7 and *lacZ* as a reporter gene. HA-pseudotypes (H5) carrying the consensus HA of the avian influenza virus isolated in Mexico, subtype H5, HA-pseudotypes (H7/06) carrying the HA subtype H7 of the influenza vaccine strain used in Mexico in 2012, and HA-pseudotypes (H7/12) carrying the HA subtype H7 of a strain responsible for the 2012 influenza outbreak in Mexico. It has been repeatedly demonstrated that HA-pseudotype titers ranging from of 10^4-10^5 RLU/mL are appropriate for neutralization assays (Wang et al., 2008, 2010). Our HA-pseudotype titers were around 10^6 RLU/mL (Fig. 1), thus showing optimal titers. We compared their stability at the temperature of a standard refrigerator (4 °C) and at the temperature of a standard freezer (−20 °C), with stability at −80 °C. The initial HA-pseudotype titers were 10^6 RLU/mL; after storage at −80 °C, HA-pseudotypes lost between 10 and 20% of their activity (Fig. 3). However, the titer remained similar to that of their fresh stocks and corresponds to that reported in the literature on pseudotypes carrying other surface proteins (Higashikawa and Chang, 2001; Wang et al., 2010; Wright et al., 2009). Comparing the initial titers of HA-pseudotypes with those of aliquots stored at 4 °C, HA-pseudotypes H5 and H7/06 lost 30% of their activity, while HA-pseudotypes H7/12 lost 44% of their activity at up to 4 weeks (Fig. 2). Although the activity decreased and demonstrated a statistically significant difference, titers of 10^5 RLU/mL can be considered optimal for neutralization assays (Higashikawa and Chang, 2001; Molesti et al., 2014; Sawoo et al., 2014; Wang et al., 2008, 2010; Wright et al., 2009). This finding is important because standard refrigerators are easy to find in common laboratories. Thus, according to our results, HA-pseudotype aliquots can be stored in the short term at 4 °C. Regarding long-term storage, HA-pseudotypes were maintained at -20 °C and titrated at a frequency of 3, 5, 9, and 12 months. H5 HA-pseudotypes lose 35% of activity at 12 months, while the remaining two HA-pseudotypes, H7/06 and H7/12, lost 48% and 54% of their activity during the same 12 months, respectively (Fig. 4). HA-pseudotype titers at -20 °C decreased around 50% at 9–12 months, revealing activity of around 10^4-10^5 RLU/mL, which was maintained until the end of storage; despite this, the remaining titer is appropriate for neutralization assays (Higashikawa and Chang, 2001; Molesti et al., 2014; Sawoo et al., 2014; Wang et al., 2008, 2010; Wright et al., 2009)), rendering this storage temperature at the long-term readily applicable in the vast majority of laboratories. The stability of HA-pseudotypes reported here support their use in countries or settings where the infrastructure and cold-chain may be unreliable, making these assays readily applicable in laboratories worldwide. The gradual loss-of-transfer of *lacZ* is probably due to pseudotype particle instability and aggregation rather than to protein degradation (Wang et al., 2010). On the other hand, similar HA-pseudotype stability among different subtypes or isolates is not expected (Molesti et al., 2012; Nefkens et al., 2007; Temperton et al., 2007; Wang et al., 2008). Loss of HA-pseudotype activity and the different survival trends observed among the different HA-pseudotypes may be due to the nature of the HA (Sawoo et al., 2014). However, the remaining activity indicated that receptor binding and transfer of the reporter gene from HA-pseudotypes into HEK 293 cells was maintained. The influence of freeze-thaw cycles on HA-pseudotype stability was evaluated by subjecting the aliquots to 1–5 cycles, the results showed a loss-of-activity of 36%, 19%, and 21% (Fig. 5) for HA-pseudotypes H5, H7/06, and H7/12, respectively; these results indicated that these HA-pseudotypes remain applicable after storage variations such as freeze-thaw cycles. Based on the data obtained in this study, H5 and H7 HA-pseudotypes were stable under different storage temperatures and freeze-thaw conditions, and they could comprise a versatile tool to use in serological techniques.

5. Conclusions

Even though HA-pseudotype activity decreased under the conditions of the different storage temperatures and freeze-thaw cycles, their titer remained optimal for application in neutralization assays. Therefore, if they are stored under the conditions tested, HA-pseudotypes can be stable and readily applicable in serological techniques.

Conflict of interest

The authors do not have associations, commercial or any other kind, that might pose a conflict of interest.

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Disclosures

The authors declare that they have no competing interests.

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