

RESEARCH REPORT

The production and development of H7 Influenza virus pseudotypes for the study of humoral responses against avian viruses

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ABSTRACT

In recent years, high pathogenicity avian influenza (HPAI) virus, H5N1, low pathogenicity avian influenza (LPAI) virus, H9N2, and both HPAI and LPAI H7 viruses have proved devastating for the affected economies reliant on poultry industry, and have posed serious public health concerns. These viruses have repeatedly caused zoonotic disease in humans, raising concerns of a potential influenza pandemic. Despite the focus on the HPAI H5N1 outbreak in 1997 some H7 strains have also shown to be occasionally adaptable to infecting humans. Therefore, applying knowledge of the H5 virus evolution and spread to the development of sensitive serological methods is likely to improve our ability to understand and respond to the emergence of other HPAI and LPAI viruses, present within the avian populations, with the potential to infect humans and other species. In the present study we describe the construction and production of lentiviral pseudotypes bearing envelope glycoproteins of LPAI and HPAI H7 avian influenza viruses, which have been responsible for several outbreaks in the past decade. The H7 pseudotypes were evaluated in pseudotype-based neutralization (pp-NT) assays in order to detect and quantify the presence of neutralizing antibodies in avian sera, which were confirmed H7 positive by inhibition of haemagglutination (HI) test. Overall, our results substantiate influenza virus pseudotype neutralization as a robust tool for influenza sero-surveillance.

KEYWORDS: HPAI, H7 lentiviral pseudotype, LPAI, micro-neutralization, pandemic potential

INTRODUCTION

Influenza A viruses that primarily cause devastating infectious disease in poultry, termed high pathogenicity avian influenza (HPAI), belong exclusively to the H5 and H7 subtypes, although not all viruses of these subtypes cause HPAI. Due to the ability of certain subtypes to mutate into a high pathogenicity form and the consequent increased mammalian transmissibility and pandemic potential (Van Kerkhove et al, 2012), several studies have also shown the alarming effects of uncontrolled long-term circulation of H7 low pathogenicity avian influenza (LPAI) viruses in poultry (Capua and Marangon, 2006). In fact, the hypothesis that

these viruses can be maintained in poultry populations and mutate to HPAI forms with public health repercussions underlines the need for monitoring systems and active surveillance (Capua et al, 2000, Belser et al, 2009). Our understanding of human infections caused by avian influenza viruses arise from numerous sources including sero-epidemiological studies, reports of human disease, relevant animal models and *in vitro* studies (Horimoto and Kawaoka, 2001, de Wit et al, 2008, Peiris, 2009). Despite the public health attention since the emergence of Asian lineage H5N1 HPAI virus outbreak in Hong Kong in 1997 and to the present day, some H7 strains have also been shown to be very adaptable for widespread infection as confirmed by several

LPAI and HPAI H7 outbreaks that have occurred worldwide in poultry, occasionally resulting in their direct transmission to humans. The current H7N3 HPAI outbreak in Jalisco, Mexico, demonstrates the continuing risk from the circulation of certain subtypes (Centers for Disease Control and Prevention, 2012). Therefore, the pandemic potential of subtype H7 viruses should not be underestimated. Hence, stressing the need for new diagnostic approaches and technologies, genetic and antigenic data should be paired for integrated “surveillance” and serological studies, specifically focusing on the development of readily applicable assays for routine testing of LPAI and HPAI viruses and newly emerged variants (Cattoli and Terregino, 2008).

To date, a comprehensive panel of influenza pseudotypes bearing HA glycoproteins belonging to several subtypes, clades and subclades have been developed and optimized (Temperton et al, 2007, Wang et al, 2008, Corti et al, 2011). This panel however comprises only a few H7 influenza viruses that have been pseudotyped for serological purposes, and therefore this study aims to show the feasibility of producing additional functional HPAI and LPAI H7 influenza pseudotypes. For this purpose, H7 pseudotypes have been produced and tested against a panel of avian sera in pseudotype-neutralization assays (pp-NT) in order to provide a tool that will offer new perspectives in avian and human influenza diagnosis, as well as renewed confirmation of their potential use for comparative serology.

MATERIALS AND METHODS

Envelope glycoprotein cloning and plasmids

cDNA for HPAI H7N3 A/chicken/Pakistan/34668/1995 (GenBank: CY035831), A/chicken/Netherlands/1/2003 (GenBank: AY338458) and LPAI H7N1 A/chicken/Italy/1082/1999 (GenBank: CY022677) was kindly provided by FAO, OIE and National Reference Laboratory for Newcastle Disease and Avian Influenza, Istituto Zooprofilattico Sperimentale delle Venezie. The H7 A/chicken/FPV/Rostock/34 was kindly provided by FL Cossett (Human Virology Department, Lyon). The HPAI A/chicken/Italy/13474/99 HA (GenBank: AJ491720) and NA were kindly provided by the FLUPAN partner laboratories.

The HA of FPV/Rostock/34 (GenBank: AAA43150) was cloned into pHCMV vector, while the HAs of HPAI H7 A/chicken/Italy/13474/99, A/chicken/Pakistan/34668/1995, LPAI H7 A/chicken/Italy/1082/1999 and A/chicken/Italy/13474/99 were cloned into pI.18, a pUC-based plasmid as described previously (Temperton et al, 2007, Wright et al, 2008). The NAs of A/chicken/Pakistan/34668/1995 (N3) (GenBank: CY035833), A/chicken/Netherlands/1/2003 (N7) (GenBank: AY340077) and N1 from A/chicken/Italy/13474/99 were also cloned into pI.18.

Serum samples

All the sera tested were provided by FAO, OIE and National Reference Laboratory for Newcastle Disease and Avian influenza, Istituto Zooprofilattico delle Venezie and comprised seven avian sera positive for antibodies to the H7N1 vaccine strain (A/chicken/Italy/1067/99 LPAI) with titres ranging from low (1:32) to high (1:2048) when tested by HI (HI reference antigen: H7N1 A/Starling/Africa/985/79)

according to standard protocols indicated by European Medicine Agency and ten sera positive for H7 collected from turkeys during an Italian outbreak of AI caused by an LPAI virus H7N3 (A/turkey/Italy/9289/V02), that were previously tested by HI (Decision2006/437/CE, 2006). Ten negative sera confirmed by Agar Gel Immunodiffusion assay (AGID) and Indirect Enzyme-linked immunosorbent assay (ELISA) (BioCheck Avian Influenza Antibody Test Kit, BioCheck UK Ltd.) were used and 2 subtyping polyclonal hyperimmune chicken antisera were used for testing subtype specificity (H7N1 and H7N3). The antisera were produced in specific pathogen-free chickens by inoculation with viruses (inactivated by beta-propiolactone if HPAI viruses) as described previously (Terregino et al, 2010). A panel of H5 positive sera that were collected from chickens vaccinated with an inactivated adjuvanted H5N2 vaccine has been included in the study.

Influenza H7 pseudotype production

A panel of lentiviral pseudotypes expressing HAs from HPAI H7N1 A/chicken/Italy/13474/99, H7N3 A/chicken/Pakistan/34668/1995, H7 A/chicken/FPV/Rostock/34, LPAI H7N1 A/chicken/1082/1999 viruses were generated essentially as described previously (Temperton et al, 2007, Corti et al, 2010, Corti et al, 2011). In order to produce HA and NA A/chicken/Italy/13474/99 influenza pseudotypes with different NAs (N1, N3 and N7), the neuraminidase activity was provided by NA plasmid co-transfection (N1 from H7 A/chicken/Italy/13474/99, N3 from H7 A/chicken/Pakistan/34668/1995 and N7 from H7 A/chicken/Netherlands/1/2003) in lieu of exogenous bacterial NA addition (HA: NA transfection ratio of 3:1) (Temperton et al, 2007). For the production of the LPAI H7 A/chicken/1082/1999 pseudotype, a TMPRSS2 protease expressing plasmid was additionally added to the transfection mixture (in order to mediate the cleavage in the LPAI H7 pseudotypes) (Wang et al, 2008). For transfection, 1×10^4 cells/well were plated 24 hr prior to the addition of a complex comprising plasmid DNA and FuGENE-6 transfection reagent (Roche, UK).

The HIV- type 1 (HIV-1) gag-pol construct (pCMV- Δ 8.91) and the reporter plasmid (pCSFLW, expressing firefly luciferase) were transfected concurrently with the required envelope glycoprotein constructs at a ratio of 1:1:1.5 ratio (p8.91:pI.18-HA:pCSFLW).

In parallel, a non-envelope glycoprotein control pseudotype was generated by co-transfection of producer cell lines with two plasmids, gag-pol pCMV- Δ 8.91 and pCSFLW. Supernatants were harvested 48 hr and 72 hr post-transfection and titrated onto HEK 293T/17 cell line.

Pp-NT neutralization assays

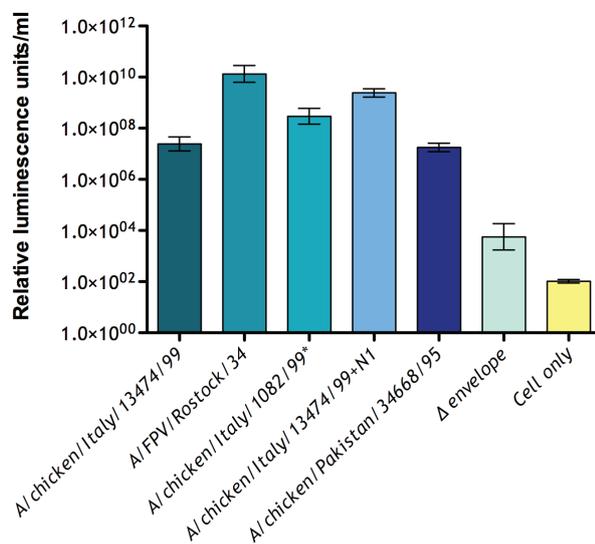
For the HPAI H7 A/chicken/Italy/13474/99, A/chicken/FPV/Rostock/34 and LPAI H7 A/chicken/Italy/1082/1999 neutralization assays, two-fold serial dilutions of each serum sample were mixed with pseudotyped viruses (resulting in 1×10^6 firefly relative light units, RLUs) at a 1:1, v/v, ratio. After incubation at 37°C for 1 hr, 1×10^4 HEK 293/17 cells were added to each well of a white 96-well-flat-bottomed tissue culture plate. For H7 A/chicken/Italy/13474/99 pseudotypes, neutralization assays were also undertaken for screening 10 H7 positive

sera collected from naturally infected turkeys and for a panel of 10 H5 positive chicken sera (previously tested by HI). RLUs were evaluated after 48 hr by luminometry using the Bright-Glo assay system (Promega, UK). To measure the neutralization activity of this panel of sera, the 90% inhibitory concentration (IC_{90}) was determined as the serum dilution resulting in a 90% reduction of a single round of infection (reporter gene-mediated signal). Values are expressed as a percentage when compared with the signal from the cell only control (equivalent to 100% neutralization and/or no infection) and the signal from a virus only control equivalent to 0% neutralization or 100% infection.

Statistical analysis

The estimation of pseudotype transduction titers (Figure 1 and Figure 2) has been performed using Excel™ software where pseudotype titers obtained at each of a range of dilution points were expressed as RLU/ml and the arithmetic mean was calculated by GraphPad Prism (version 5, GraphPad Software, San Diego, CA, USA). Statistical analyses were also undertaken for the analysis of pp-NT assays using GraphPad. Pp-NT titers were normalized and IC_{90} values were calculated by a non-linear regression model (log (inhibitor) vs normalized response-variable slope) analysis (Table 1).

H7 HPAI and LPAI pseudotypes



* Low pathogenic avian influenza (LPAI) H7

Figure 1. Infectivity of pseudotyped lentiviral vectors carrying HA glycoprotein of HPAI A/chicken/Italy/13474/1999, A/chicken/Pakistan/34668/1995, H7 A/chicken/FPV/Rostock/34 and LPAI H7N1 A/chicken/1082/1999. Influenza pseudotypes were produced by adding exogenous neuraminidase 24 hr after transfection (for H7 A/chicken/Italy/13474/1999, additionally an NA plasmid has been used). The transduction titers of the released pseudotypes were expressed as the mean \pm SD of relative luciferase units (RLUs).

RESULTS

Production of lentiviral HPAI and LPAI H7 pseudotype particles (H7pp)

Pseudotypes expressing HAs from four H7 viruses (HPAI A/chicken/Italy/13474/1999, A/chicken/FPV/Rostock/34, A/chicken/Pakistan/34668/1995 and LPAI H7 A/chicken/1082/1999) were generated (Figure 1). Additionally, pseudotypes bearing both HA and NA glycoproteins of H7N1 A/chicken/Italy/13474/99 were generated (Figure 1). We evaluated the transduction efficiency of each H7 pseudotype by measuring the titers expressed as RLU/ml in HEK293/17 cells. For all the transduction measurements two controls were included: a non-envelope glycoprotein control and a cell only control.

As expected, the RLU values for the non-envelope control (pCMV- Δ 8.91 and the reporter pCSFLW plasmids) were similar to mock transduction. In contrast, significantly higher RLU values, compared to the controls, were detected in cells transduced with HPAI H7 A/chicken/Italy/13474/99, A/chicken/FPV/Rostock/34, A/chicken/Pakistan/34668/1995 and LPAI H7 A/chicken/1082/1999 pseudotypes. Interestingly, in cells transduced by H7N1 A/chicken/Italy/13474/99 pseudotypes (where the NA activity was provided by the cognate plasmid) the RLU values were two \log_{10} s higher than the same H7 HA pseudotype produced by exogenous NA treatment.

Subsequently, a set of three HPAI H7 A/chicken/Italy/13474/99 HA+NA pseudotypes with NAs belonging to different H7 avian influenza strains (N1, N3, N7)

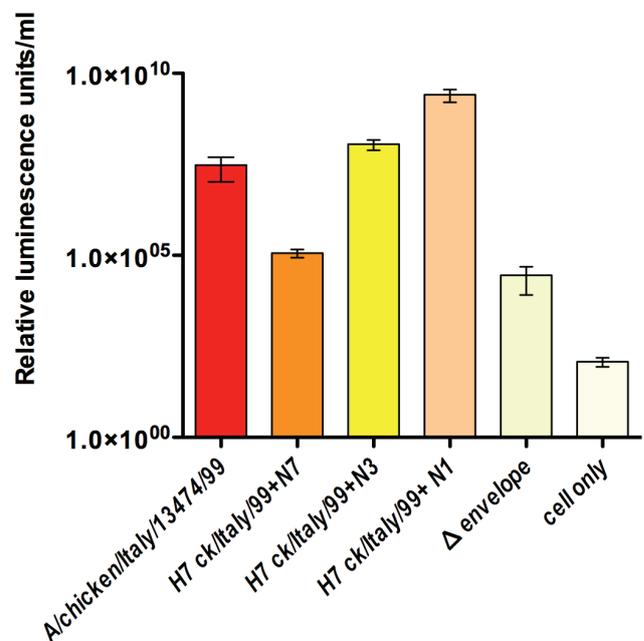


Figure 2. Panel of H7 A/chicken/Italy/13474/1999 HA pseudotypes produced in combination with different NA plasmids (N1 from A/chicken/Italy/13474/1999, N3 from A/chicken/Pakistan/34668/1995 and N7 from A/chicken/Netherlands/1/2003). Comparison of transduction efficiency in target HEK 293T cells. Titers of released pseudotypes were expressed as the mean \pm SD of relative luciferase units (RLUs).

Table 1. Comparison of neutralization activity of chicken immune sera (positive for the H7N1 vaccine strain: A/chicken/Italy/1067/1999) against three different H7 pseudotypes: A/ck/Italy/1082/1999, A/ck/Italy/13474/1999 and A/chicken/FPV/Rostock/34 (Set A). H7 Influenza pseudotypes bearing HA from A/ck/Italy/13474/1999 were also used for testing neutralizing activity of a panel of sera (Set B; No. 8–No. 17) collected from naturally infected turkeys (positive for H7N3 A/turkey/Italy/9289/V02). Additional sera previously found H5 positive by HI have been tested (Set C). A non-linear regression approach, dose-response inhibition has been used for the analysis). Values are reported as 90% inhibitory concentration (IC₉₀). Haemagglutination inhibition titres are also reported.

Serum No.	(A) H7 positive sera (vaccinated with an LPAI H7N1 vaccine)				(B) H7 positive sera (H7N3 positive, naturally infected)			(C) H5 positive sera (vaccinated with an LPAI H5N2 vaccine)		
	IC90 vs H7N1 A/ chicken/1082/ 1999 (LPAI)	IC90 vs H7N1 A/ chicken/13474/ 1999 (HPAI)	IC90 vs A/FPV/ Rostock/34	HI titres A/ck/Italy/ 1067/99H7N1	Serum No.	IC90 vs H7N1 A/ chicken/13474/ 1999 (HPAI)	HI titres A/ty/Italy/9289/ V02 H7N3	Serum No.	IC90 vs H7N1 A/ chicken/13474/ 1999 (HPAI)	HI titres A/ty/Italy/ 80H5N2
1 (6612-36)	>5120	>5120	>5120	1:2048	8	>5120	1:2048	3929-1	40	1:2048
2 (6612-42)	>5120	>5120	>5120	1:1024	9	>5120	1:1024	3929-9	40-80	1:1024
3 (6612-45)	>5120	>5120	>5120	1:512	10	2560-5120	1:512	3929-6	<40	1:512
4 (188-2)	1280-2560	320-640	640-1280	1:256	11	160-320	1:256	3930-19	<40	1:256
5 (188-1)	1280-2560	640-1280	640-1280	1:128	12	640-1280	1:128	3930-20	<40	1:128
6 (10-3)	2560-5120	320-640	320-640	1:64	13	160-320	1:64	3933-44	<40	1:64
7 (10-4)	1280-2560	160-320	160-320	1:32	14	80-160	1:32	3933-41	<40	1:32
H7N1 +ve	27782	>5120	>5120	n/d	15	<40	1:16	3933-50	<40	1:16
H7N3 +ve	>5120	>5120	640-1280	n/d	16	40-80	1:8	3933-41	<40	1:8
					17	<40	1:4	3933-44	<40	1:4

was generated and transduction efficiency was estimated. Surprisingly, low titers were obtained when the N7 plasmid (from H7 A/Netherlands/1/2003) was used compared to N3 and N1 plasmids for H7 A/chicken/Italy/13474/99 pseudotype production (4 log₁₀s higher when the N1 plasmid was used instead of the N7 plasmid) (Figure 2).

Development and evaluation of H7 pseudotype-based neutralization assay

To develop and evaluate the utility of H7 pseudotypes in pp-NT assays, neutralizing antibody responses were measured in sera obtained by vaccination of chickens with a H7N1 vaccine against three different H7 pseudotypes: HPAI H7 A/chicken/FPV/Rostock/34, A/chicken/Italy/13474/99 and LPAI H7 A/chicken/1082/1999 strains. The same panel of sera was also found positive when tested by HI against H7N1 A/Starling/Africa/985/79.

The results obtained using all the H7 pseudotypes when tested in duplicate displayed a clear and wide antibody response confirmed by high neutralization titers (titers between $\geq 1:320$ and $>1:5120$) compared to the negative controls (data not shown). The neutralization titers for H7 LPAI A/chicken/1082/1999 were slightly lower (Table 1A) compared to HPAI H7 A/chicken/FPV/Rostock/34 and A/chicken/Italy/13474/99 pseudotypes (Table 1B, 1C). Additional pp-NT assays have been undertaken to test H7 A/chicken/Italy/13474/99 pseudotypes against sera from H7 positive turkeys (by natural infection) and H5 positive chickens (by vaccination) and results are shown in Table 1.

DISCUSSION

Within the last decade, poultry outbreaks caused by LPAI and HPAI viruses of the H7N1, H7N2, H7N3, H7N4 and H7N7 subtypes have resulted in the culling of millions of birds. Since 2002, this has resulted additionally in >100 cases of human exposure in the Netherlands, Italy, Canada, the United States and United Kingdom (Capua and Marangon, 2006, Belser et al, 2009). The presence of H7 LPAI viruses in many countries and the current H7N3 outbreak in Mexico demonstrates the constant risk posed by certain H7 avian influenza subtypes to mutate from the low pathogenicity to the high pathogenicity form. Despite the relatively limited human transmissibility of HPAI H7 viruses, they have been shown to share many properties and molecular features with the HPAI H5 subtype. Primarily, the presence of a polybasic cleavage site within the HA sequence leads to the recognition by ubiquitous proteases and subsequent systemic spread of the virus resulting in death of infected birds. Additionally, the marked increase in the number of LPAI outbreaks highlights the importance of carrying out an active sero-surveillance for this subtype in order to monitor the evolution of these strains have the potential to impact on human and animal health (Capua and Alexander, 2007).

With a serological perspective, this study aimed to develop a panel of H7 HPAI and LPAI influenza pseudotypes from strains that were responsible for severe outbreaks, and to

employ them as tools in serological assays in parallel with the classical serological method HI.

A panel of functional HPAI H7 pseudotypes was produced in addition to a LPAI H7 pseudotype, overcoming the difficulties in cleaving the precursor HA from a strain that lacks the polybasic cleavage site (Wang et al, 2008). For all the H7 pseudotypes, high transduction titers were obtained as confirmed by RLU/ml values in Figure 1. However, the A/chicken/Pakistan/34668/1995 strain exhibited a lower transduction efficiency. This is possibly due to lower glycoprotein expression or sub-optimal cleavage of the HA by cellular proteases (Nefkens et al, 2007, Wang et al, 2008, Choi et al, 2009, Klenk and Garten, 1994). Production of H7 pseudotypes bearing HA alone from A/chicken/Italy/13474/99, or HA+NA by additional co-transfection of different NAs was attempted. The rationale for this is the observation that H7 subtype viruses with many different NA types have transmitted from birds to humans compared to the situation with the H5 subtype (Belser et al, 2009, Abdel-Ghafar et al, 2008). We show using our pseudotype production system that not all the HA and NA combinations were equally effective at generating high-titer virus stocks (Figure 2).

Lastly, three H7 pseudotypes were used for neutralization assays and results compared to the classical HI serological test. As expected, the pp-NT assay returned higher titers compared to HI and a significantly lower antibody response was detected against HPAI strains compared to the LPAI strain.

CONCLUSIONS

- The development of pseudotype influenza viruses may provide excellent serological reagents for testing sera originating from birds but also from other species, including humans.
- The flexible and safe use of pseudotype influenza virus in neutralization assays may prove useful for influenza control strategies enabling the evaluation of the levels of neutralizing immunity induced by field isolates and vaccines, and the possible mechanisms of cross-reactive immunity.

COMPETING INTERESTS

None declared.

LIST OF ABBREVIATIONS

AGID; Agar Gel Immunodiffusion assay
 ELISA; Enzyme-linked immunosorbent assay
 HA; Haemagglutinin
 HI; Inhibition of haemagglutination
 HPAI; High pathogenicity avian influenza
 LPAI; Low pathogenicity avian influenza
 NA; Neuraminidase
 PP-NT; Pseudotype-based neutralization
 RLUs; Relative luminescence units

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