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Comparative efficacy of North American and antigenically matched reverse genetics derived H5N9 DIVA marker vaccines against highly pathogenic Asian H5N1 avian influenza viruses in chickens

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ABSTRACT

Highly pathogenic (HP) H5N1 avian influenza has become endemic in several countries in Asia and Africa, and vaccination is being widely used as a control tool. However, there is a need for efficacious vaccines preferably utilizing a DIVA (differentiate infected from vaccinated animals) marker strategy to allow for improved surveillance of influenza in vaccinated poultry. Using a reverse genetics approach, we generated Asian rgH5N9 vaccine strain deriving the hemagglutinin gene from A/chicken/Indonesia/7/2003 (H5N1) with modification of the cleavage site to be low pathogenic (LP) and N9 neuraminidase gene from the North American LP A/turkey/Wisconsin/1968 (H5N9) virus. The recombinant rgH5N9, A/turkey/Wisconsin/1968 (H5N9) A/chicken/Hidalgo/232/1994 (H5N2), and wild type HP A/chicken/Indonesia/7/2003 (H5N1) viruses were used to prepare inactivated oil-emulsified whole virus vaccines. Two weeks after vaccination, chickens were challenged with either Asian HP H5N1 viruses, A/chicken/Indonesia/7/2003 (W.H.O. clade 2.1) or A/chicken/Supranburi Thailand/2/2004 (W.H.O. clade 1.0). The H5 HA1 of the North American vaccine strains exhibited 12% amino acid differences including amino acid changes in the major antigenic sites as compared to the Asian HP H5N1 challenge viruses, serologically exhibited substantial antigenic difference, but still provided 100% protection from mortality. However, challenge virus shedding was significantly higher in chickens immunized with antigenically distinct American lineage vaccines as compared to the antigenically matched Asian rgH5N9 and the wild type Asian H5N1 vaccine. The antibody response to the heterologous subtype neuraminidase proteins were discriminated in vaccinated and infected chickens using a rapid fluorescent 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid sodium salt as substrate for neuraminidase inhibition assay. This study demonstrates the value of using a vaccine containing antigenically matched H5 hemagglutinin for control of HP H5N1 avian influenza in poultry and the potential utility of a heterologous neuraminidase as a DIVA marker.

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1. Introduction

Influenza A viruses have been isolated from many different avian and mammalian species, but wild waterfowl and shorebirds are thought to be the primary reservoir of influenza A virus in nature [1]. The influenza A virus genome consists of eight strands of negative sense RNA, which codes for 10–11 different proteins [2–5]. Influenza A viruses are characterized into distinct antigenic subtypes based on their two surface glycoprotein's, the hemagglutinin (HA) and the neuraminidase (NA) [6]. Influenza A viruses representing 16 HA and 9 NA antigenic subtypes have been isolated from avian species, and can theoretically be found in any combination of subtypes (e.g. H1N1, H5N9 etc.) [1,7]. The HA protein is initially synthesized as a single polypeptide precursor (HA0), which is then cleaved by host proteases into HA1 and HA2 subunits [8,9]. The clinical disease outcome in influenza A virus infection among avian species largely depends upon the ability of different cellular proteases to cleave the hemagglutinin protein. The presence of polybasic amino acids or an insert of amino acids at the HA cleavage site of H5 and H7 subtype viruses are characteristic of highly pathogenic (HP) viruses which by definition are highly lethal for chickens in standard animal pathotyping studies. Low pathogenic (LP) H5 and H7 viruses are distinctive in that they can quickly and unpredictably change to HP viruses with amino acid changes at the cleavage site [1,8]. In addition to the evolution of antigenic subtypes, avian influenza A viruses have evolved

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into distinct genetic lineages within the HA subtypes, Eurasian and North American lineages, presumably as a result of the predominant north to south migration pattern of their waterfowl hosts [1,11].

Highly pathogenic H5N1 avian influenza viruses cause a serious disease in poultry and is a notifiable disease to the Office International des Epizootics, the World Organization of Animal Health, and therefore outbreaks adversely affect international trade of poultry and poultry products [9]. An Asian lineage HP H5N1 avian influenza strain that emerged in domestic geese in southern China during 1996 [10] became endemic in the region, and continued to spread to other parts of the country and across Asia [11,12]. Beginning in late 2003, an unprecedented number of outbreaks of this H5N1 lineage occurred in many Asian, African and European countries [13,14]. Recent W.H.O. antigenic and phylogenetic analyses criteria distinguish Asian lineage H5N1 viruses into nine major clades, indicating the array of genetic and antigenic diversity among this lineage of avian influenza viruses, underscoring a challenge posed to control H5N1 avian influenza in poultry [15]. The zoonotic transmission of the HP H5N1 avian influenza virus strains from poultry to humans in Asian and African countries resulted in fatal illnesses in 241 of 383 confirmed cases and continue to pose a serious threat to the public health [16]. Because of the repeated outbreaks of HP H5N1 avian influenza in poultry in Asia and Africa, and associated human infections [17–22], control of the disease in poultry has become a high priority. Traditional control strategies against HP H5N1 avian influenza outbreaks in poultry include the identification and slaughter of affected and at risk poultry [23]. However, eradication by slaughter may be prohibitively costly when the outbreak is widespread within a country or region. Vaccination as a control tool has been gaining favor as a potentially more cost effective approach for controlling the virus, reducing the economic loss to poultry farmers, and contributing to improved food security in developing nations. Although several different types of vaccines are available worldwide, vaccination of poultry is primarily done with whole virus inactivated oil-emulsified vaccines that when properly administered can provide high antibody levels to the HA protein and result in significant reductions of viral shedding. Vaccination has been useful for the control of HP H5N1 avian influenza in chickens in Hong Kong [24] and H5N2 avian influenza in Mexico, although vaccination did not eradicate the LP H5N2 virus in Mexico [25].

One disadvantage of the inactivated whole virus vaccines is they affect serologic surveillance when using the common influenza type A specific diagnostic tests like the agar gel immunodiffusion test (AGID) or the commercially available enzyme linked immunosorbent assay, because both vaccinated and naturally infected birds develop antibody to both the nucleoprotein and matrix proteins which are the primary antigens for these tests. Ideally any vaccine approach would benefit from a DIVA (differentiate infected from vaccinated animals) strategy where an easy and cost effective serologic test can provide surveillance even in vaccinated flocks [23,26]. One proposed DIVA strategy is the heterologous neuraminidase (hNA) approach where the hemagglutinin subtype of the vaccine is matched to the predominantly circulating field strain, but the neuraminidase subtype is different from the field strain. Therefore the presence of antibodies or lack of antibodies to the neuraminidase protein can determine if a bird was infected, vaccinated or vaccinated and then infected with avian influenza virus. Inclusion of a rarely circulating NA subtype in poultry influenza virus such as N4, N5, N6, N8 and N9 in the vaccine, either a naturally occurring strain or in an engineered vaccine strain, can provide a useful and effective DIVA marker, and reduces the possibility of confusion if more than one subtype of avian influenza is circulating at one time [27,28]. The European Union has approved use

Table 1
Antigenic relatedness (cross-HI antibody titers) of North American and Asian H5 avian influenza A viruses used in vaccine and challenge experiments.

Immune anti-serum[a]	Viruses used as antigen[b]				
	A/turkey/Wisconsin/1968 (H5N9)	A/chicken/Hidalgo/232/1994 (H5N2)	A/chicken/Indonesia/7/2003 (rgH5N9)	A/chicken/Indonesia/7/2003 (wtH5N1)	A/chicken/Supranburi Thailand/2/2004 (wtH5N1)
A/turkey/Wisconsin/1968 (H5N9)	**256**[c]	128	32	32	32
A/chicken/Hidalgo/232/1994 (H5N2)	64	**128**	32	32	16
A/chicken/Indonesia/7/2003 (rgH5N9)	32	32	**256**	256	128
A/chicken/Indonesia/7/2003 (wtH5N1)	32	16	128	**128**	128
A/chicken/Supranburi Thailand/2/2004 (wtH5N1)	32	32	128	128	**256**

wt: wild type virus; rg: reverse genetics derived mutated LPAI hemagglutinin cleavage site amino acid sequence RETR/GLF containing virus.

[a] Immune anti-serum was produced by single dose s/c vaccination of 2-week-old SPF white Leghorn chickens with 0.5 ml of oil-emulsion vaccine. The serum samples from individual chickens were collected 3 weeks after immunization. Cross-HI assay was performed using immune serum from a single bird against each virus strain.

[b] Influenza virus was inactivated using β-propiolactone and used as antigen in the micro-titer HI assay. Four HA units of viral antigen was used in the HI assay.

[c] Endpoint HI titers with homologous virus strain (bold values) and heterologous H5 virus strains (non-bold values) in two-fold diluted serum samples.