Antigenically and Genetically Diverse Isolates of Low Pathogenicity H9N2 Avian Influenza Virus Provide Cross-Clade Vaccinal Protection

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Abstract

Avian influenza viruses, H9N2 subtype, are endemic in Asia and the Middle East. The Israeli H9N2 (G1 lineage) consists of five phylogenetic clades that were detected in the country since the year 2000. The influence of the inter-clade genetic differences on vaccine efficacy was evaluated by vaccination-challenge trials in specific pathogen free (SPF) chickens. Experimental vaccines utilizing isolates belonging to Israeli H9 clades IV and V were prepared with Montanide ISA 70VG adjuvant. When challenged with H9 AIV strains from different genetic clades and antigenic groups provided similar protection, indicating the existence of cross-genetic clade protection (i.e. reduction in amount of virus shed and number of chickens shedding virus). Notably, the protection conferred by both experimental vaccines was highly dependent on the infection dose of the challenge virus.

Keywords: Low pathogenic avian influenza virus, H9N2 influenza, poultry disease, cross-clade protection, quantitative real-time RT-PCR

Abbreviations

AIV = avian influenza virus; DPI = days post inoculation; EID_{50} = 50% egg infectious dose; HA = hemagglutinin; HI = hemagglutinin inhibition; KVI – Kimron Veterinary Institute; qrRT-PCR = quantitative reverse-transcription real time polymerase chain reaction; SPF = specific pathogen free; PI = protection index

Introduction

In Israel, H9N2 avian influenza viruses (AIVs) have been isolated and characterized molecularly since the year 2000. All AIV H9N2 isolates belong to the G1 lineage, possess a cleavage site characteristic to low pathogenic AIV and were classified into five phylogenetic clades [6,7]. The first and second AIV H9N2 clade was prevalent from May 2000 to April 2003, the third circulated from April 2003 to December 2006, while afterwards a fourth genetic clade, subdivided into four sub-clades, (A-D) appeared in the years 2006 (A), 2007 (B), 2010 (C) and 2013

(D) and has been observed sporadically. Between the years 2011 and 2013 a fifth clade was prevalent in Israel. The isolates A/turkey/Israel/965/02 and A/chicken/Israel/215/07, belonging to the 2nd and 4th genetic clades, respectively, were selected as prototype vaccine strains, and were in use from 2003 to 2008, and from 2008 until today, respectively. However, an additional H9N2 vaccine, based on a clade V isolate, was also made available for commercial use.

The genetic richness and plasticity of AIV H9N2 in the Middle East region inspired the phylogenetic analysis of the AIV H9N2, including 123 H9 Israeli and 180 international Middle East isolates [7]. The main findings revealed: (a) the most recent common ancestor (tMRCA) occurred 3 months before first isolation in Israel; (b) the genetic diversity peaked twice, corresponding in time to the appearance of clades III and IV and to the introduction of the two vaccines; (c) the mean evolution rate was 6.123 E-3 substitutions/site/year, similarly to countries that use AIV vaccination.

The selection process and decision on which vaccine virus would be the best fit, is the most complex stage in vaccination to AIV H9N2 [11]. While the phylogenetic relatedness of the HA gene and protein is the basic selection criteria, the method for antigenic matching by antigenic cartography (AC) established by Smith et al, [9] opened a new era in vaccine virus selections and offers an additional means for evaluating candidate vaccine strains. Antigenic cartography provided a means to evaluate the antigenic relatedness of current field isolates compared to a vaccine virus and has been used with the H9N2 isolates from Israel [19]. Recently a method that integrates phylogeny and antigenic cartography has been developed and applied successfully for swine influenza virus classification [3]. However, the most reliable way to select the vaccine virus strain in terms of the protection afforded to the bird, is by *in vivo* evaluation of efficacy with vaccination-challenge trials.

In view of the widespread AIV H9N2 in the Middle East and its extensive genetic evolution, the present study evaluates

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several phylogenetically and antigenically diverse H9 isolates for differences in vaccine protection (i.e. reduction in shed, increased infectious dose) against diverse H9 challenge isolates.

Materials and Methods

Isolates

AIV isolates were propagated in embryonated chicken's eggs by standard procedures. The AIVs were grown and amplified by inoculation in 9-10 day-old SPF (specific pathogen free) (SPAFAS, U.S.A.) embryonated eggs [8, 14]. The virus titers were first determined in the original allantoic fluid (AF), and then ten-fold dilutions in sterile PBS were used for inoculation in order to calculate the 50% embryo infection dose$_{50}$ (EID$_{50}$) by the method of Reed and Munch (18). The AIV H9N2 isolates, used in the present study are: A/Ostrich/Israel/1436/2003 (H9N2) clade I+II (Os/1436), A/Chicken/Israel/215/2007 (H9N2) clade IVA (Ck/215) and A/Chicken/Israel/1163/2011 (H9N2) clade V (Ck/1163).

Vaccines

Vaccines were prepared using AIV antigen inactivated with 0.02% formalin from infectious egg fluids. After inactivation the allantoic fluids were inoculated sequentially twice, to ensure inactivation. An experimental emulsion was prepared on the day of vaccination using a commercial oil adjuvant, Montanide ISA 70VG (Seppic, Inc. Paris, France), in accordance with the manufacturer's instructions. Vaccines were standardized for a dose of 512 hemagglutinating units per dose in a volume of 0.5 ml per bird.

Vaccination-challenge studies

Based on the antigenic map of the Israeli H9N2 isolates (19) three isolates were selected for vaccine studies: Os/1436) (genetic clade I+II), Ck/215, (genetic clade IVA); and Ck/1163 (genetic clade V). The isolates were selected to provide antigenic and genetic (6) diversity. In trial 1, the pilot vaccination-challenge trial, two experimental vaccine preparations, Ck/215 and Ck/1163 were evaluated for their ability to protect against all three challenge viruses at the dose of 10^6 EID$_{50}$ per bird. A second more comprehensive trial, trial 2, was conducted with Ck/1163 challenge at four doses, ranging from 10^3 to 10^6 EID$_{50}$ per bird in 10-fold increments. Each trial included unvaccinated challenge controls.

In both trials SPF chickens were vaccinated by the subcutaneous route at three weeks of age and challenged at three weeks post vaccination by the intrachoanal (ICh) route. The ICh challenge administration simulates natural upper respiratory exposure, as the virus is administered into the middle nasal cavity through the choanal cleft, ensuring that each bird received the full dose [13]. To verify that the chickens were free of AIV infection, immediately prior to vaccination oro-pharyngeal (OP) swabs were collected to evaluate virus shed by qrRT-PCR, and the birds were bled to detect AIV antibodies by HI. The birds were bled twice during the trials, just before the challenge, to evaluate the antibody following vaccination, and 10 days post challenge, to evaluate the secondary humoral response.

Since experimental infection of SPF chicks in isolators with LPAIV does not cause clinical disease, vaccination efficacy was measured by comparing the replication of the challenge virus (i.e. virus shed) between vaccinated and unvaccinated controls. To evaluate the vaccine efficacy oropharyngeal (OP) swabs were collected at 2 and 4 days post-inoculation (dpi) and virus shed was evaluated by qrRT-PCR by virus gene copy number. The protection index (PI) was also calculated according to the formula: protection index = the percentage of AIV positive birds that were unvaccinated and challenged minus the percentage of positive birds in the vaccinated and challenged group divided by the percentage of positive birds in the unvaccinated and challenged control group x 100 (20).

Differences in the numbers of chickens shedding detectable levels of virus were analyzed by Fisher's exact test. In trial 1 the significance of differences of mean gene copy numbers between vaccines on the same day with the same challenge virus were determined by one-way RM ANOVA. In trial 2 differences in shed of gene copy number between groups were analyzed by ANOVA on ranks (Dunn's test was used with unequal group sizes) (Sigma plot, 12.0, Systat Software). A p value of 0.05 or less was considered significant.

Hemagglutination inhibition assay

Hemagglutination inhibition (HI) assay was conducted in accordance with standard procedures [17]. Briefly, 2-fold serial dilutions of 25 μl of serum were made in 25 μl of phosphate buffered saline (PBS). Diluted sera were incubated for 30 min at room temperature with 4HAU/25 μl of antigen, and then25 μl of 1% chicken red blood cells were added. The test was evaluated after 30 min of incubation at room temperature. Titers were calculated as the reciprocal of the last HI positive serum dilution and samples with HI titers of 3 or below were considered negative.

Quantitative rRT-PCR

Viral RNA extractions for the quantitative (qrRT-PCR) assays were extracted using the QIAamp® Viral RNA Mini Kit (250) QIAGEN Ltd., Israel, in accordance with the manufacturer's instructions. Quantitative rRT-PCR which targets the influenza virus M gene [4,10] was performed using the StepOnePlus Real-time PCR System (Applied Biosystems, Foster City, CA). The amplification mix at a volume of 20 μl, contained 10 μl of Mastermix (qScript XLT One-step RT-qPCRTough Mix, ROX, Quanta BioSciences, Inc. Gaithersburg, MD, U.S.A.), 10 μM of each primer, and 5 μM probe, 2 μl of the RNA sample. The cycling conditions were: cDNA synthesis for 10 min at 50^0C, denaturation for 5 min at 95^0C and 40 cycles of 10 sec. at 95^0C and 45 sec at 60^0C. The quantitation of viral particles was based on the copy number calculator described by Tan and Tannock [16] based on the TOPO TA plasmid (Invitrogen, Ltd., USA) and the AIV M gene amplicon. The quantitative analysis of amplicon number, as represented in the reaction by plasmid number reflects the number of gene copies in the reaction mix. For calculation of the relative copy number of each amplicon, in each qrRT-PCR reaction a fresh preparation of plasmid DNA, each at defined copy numbers of

1,000,000, 10,000 and 100 amplicon-containing plasmids was used in each assay. The lowest level of detection was between 10 and 100 molecules, therefore the level of detection was determined as 100 molecules. A bird was considered positive if the C_T value by qrRT-PCR was below 35.3 and at least 100 virus particles were detected in the reaction.

Results and Discussion

In trial 1 there were no significant differences between the vaccines in the number of birds shedding virus or the mean gene copy numbers detected in oral swabs for any challenge virus at either 2 or 4 DPI (Table 1). However, there were differences between challenge viruses indicating that Ck/215 and Ck/1163 were replicated better than Os/1436. Since Os/1436 is an ostrich isolate, it may be less well adapted to chickens. Importantly, neither vaccine eliminated infection or virus shed.

In the second trial, which was more comprehensive than trial 1, protection was compared using different doses. At 2DPI there was no difference in the number of birds shedding except in the Ck/1163 vaccine group at the 10^5 EID_{50} per bird dose group which was significantly lower than non-vaccinates and Ck/215 vaccinated chickens (Table 2). Similarly, at 4DPI the number of chickens shedding was significantly lower in the Ck/1163 vaccinated group at both the 10^4 and 10^5 EID_{50} per bird doses. No statistical differences were observed at time in the gene copies detected in oral swabs (too few birds were positive for shed at the lowest 2 doses for statistical analysis). These data suggest that there is marginally better protection with the homologous vaccine-challenge virus, but at low and high doses this difference disappears either because infection levels are too low for detection or too high and may overwhelm the vaccine protective response. Moreover, isolate Ck/1163 replicates systemically, as evidenced by rRT-PCR and by histology (Davidson, unpublished data).The dose effect is reflected in the PI, which shows a trend toward better protection from shed at lower doses.

It should be noted that inactivated vaccines induce a serum antibody response, but no T-cell response and minimal secretory antibody, so the vaccine will limit the replication of LPAIV less well than highly pathogenic AIV. However, LPAIV strains will vary in infectivity, as seen with Os/1436, which does not seem to be adapted to chickens. Conversely, there may be field isolates which replicate very well in chickens. Here, serology was conducted to confirm the response to vaccination (21 days post vaccination) and after challenge. No antibody was detected in the non-vaccinated birds and all vaccinated birds responded to the vaccine. No differences were noted between both viruses in their immunizing and antigenic properties. Increases in antibody titers post challenge confirm infection status.

Antigenic cartography has been used previously for the prediction of the most appropriate vaccine virus against highly pathogenic AIV, including H7 [1, 12] and H5N1 [2] strains. Since the positive predictive value of antibody alone for protection [15] seen for highly pathogenic AIV is probably not the same for low pathogenic AIV we explored antigenic mapping for its effectiveness in with low pathogenicity H9N2. H9N2 is of critical importance in the Middle East and it was previously noted that H9N2 isolates acquired a higher replication and horizontal spread ability *in vivo* as they continued to circulate in poultry [5]. Although, the differences between the non-vaccinated groups and vaccinated chickens were not significant here, in the field the utility of vaccine is evident. Therefore, differences which may not be notable with small SPF groups are amplified in the field due to higher stress conditions for the birds.

In effort to offer antigenic cartography on H9 AIV [19] to complement phylogenetics [7] as a refined approach for the selection of the most tailored vaccines to AIV H9N2 we conducted several vaccination-challenge studies to evaluate this approach. Our main findings indicate that despite the phylogenetic and antigenic difference between the AIV H9N2 clades, protection

Table 1: Number of chickens positive for virus shed and mean amount of virus shed after challenge with 10^6 50% egg infectious doses per bird of A/chicken/Israel/1163/2011 H9N2(Ck/1163). Inactivated vaccines were prepared with A/chicken/Israel/215/2007 H9N2 (Ck/215) and Ck/1163. Statistical significance for the number of chickens positive for virus shed for a specific day post infection was determined by the Fisher exact test. The significance ofdifferences of mean gene copy numbersbetween vaccines on the same day with the same challenge virus were determined by one-way RM ANOVA. A p value of ≤ 0.05 was considered significant for both tests.

Day post inoculation	Challenge virus	Vaccine virus: Ck/215		Vaccine virus: Ck/1163	
		No. positive/ Total (%)*	Mean gene copies detected per positive bird	No. positive/ Total (%)*	Mean gene copies detected per positive bird
2	Os/1436	0/10[a] (0)	Not detected	0/10[a] (0)	Not detected
	Ck/215	7/10[b] (70)	3.8X 10^{4a}	9/11[b](81)	2.1x10^{5a}
	Ck/1163	5/6[b] (83)	5.6x10^{4a}	5/9[b] (56)	4.8x10^{3a}
4	Os/1436	2/10[a] (20)	2.0x10^3	4/10[ab] (40)	3.1x10^3
	Ck/215	4/10[ab] (40)	6.9x10^{4a}	9/11[b] (81)	2.1x10^{5a}
	Ck/1163	6/6[b] (100)	4.9x10^{5a}	6/9[ab] (67)	2.6x10^{4a}

*AIV positive by real-time RT-PCR = C_T < 35.3 and copy no. > 100 (percent of birds positive in group)
a, b, c in upper superscript - statistical differences are noted by different super scripts within the same day post infection. The absence of superscripts indicates that insufficient chickens were positive to conduct reliable statistical analysis of virus shed levels.

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Table 2: Number of chickens shedding virus orally, mean gene copy numbers by group, and protection index (PI) with non-vaccinated chickens, and chickens vaccinated with inactivated vaccines prepared with A/chicken/Israel/215/2007 H9N2 (Ck/215) and A/chicken/Israel/1163/2100 H9N2 (Ck/1163) against various doses of Ck/1163low pathogenicity avian influenza virus. Statistical significance for the number of chickens positive for virus shedfor a specific dose and day post infections were determined by the Fisher exact test and the significance of differences in gene copy number was determined by ANOVA on ranks, Dunn's test (insufficient birds were positive for shed in the 10^3 and 10^4 EID_{50} dose groups to evaluate). A p value of ≤ 0.05 was considered significant for both tests. Statistical differences are noted by different super scripts within a dose group and day post infection. In the 10^3 and 10^4 EID_{50} dose groups the absence of superscripts indicates that insufficient chickens were positive to conduct reliable statistical analysis of virus shed levels.

Day post inocula-tion	Challenge dose EID_{50}	No vaccination		Vaccine virus: Ck/215				Vaccine virus: Ck/1163			
		No. positive/Total (%)*	Mean gene copies detected per positive bird	No. positive/Total (%)	Protection Index**	Mean gene copies detected per positive bird	Fold change vs non vaccinated birds‡	No. positive/Total (%)	Protection Index	Mean gene copies detected per positive bird	Fold change vs non vaccinated bird c
2	10^3	1/5a (20)	2.2×10^2	1/6a (17)	15	3.2×10^3	14.0	1/9a (11)	45	7.9×10^2	3.6
	10^4	2/5a (40)	8.4×10^3	3/6a (50)	NA†	1.3×10^4	1.5	0/9a (0)	100	Not detected	NA
	10^5	5/5a (100)	3.3×10^{4a}	3/6a (50)	50	6.0×10^{4a}	1.8	1/9b (11)	89	1.6×10^{4a}	0.5
	10^6	5/5a (100)	6.5×10^{4a}	5/6a (83)	17	5.6×10^{4a}	1.0	5/9a (55)	45	4.8×10^{3a}	0.07
4	10^3	1/5a (20)	1.3×10^6	3/6a (50)	NA	1.6×10^4	0.01	0/9a (0)	NA	Not detected	NA
	10^4	4/5a (80)	6.9×10^5	5/6a (83)	NA	4.6×10^5	0.7	0/9b (0)	NA	Not detected	NA
	10^5	5/5a (100)	1.2×10^{6a}	5/6a (83)	17	1.4×10^{5a}	0.12	2/9b (22)	78	2.2×10^{3a}	0.002
	10^6	5/5a (100)	1.0×10^{6a}	6/6a (100)	0	4.9×10^{5a}	0.49	6/9a (67)	33	2.6×10^{4a}	0.03

*.AIV positive by real-time RT-PCR = CT < 35.3 and copy no. >100 (percent of birds positive in group)

** Protection Index = The percentage of AIV positive birds of unvaccinated + challenged controls less the percentage of positive birds in the vaccinated + challenged group divided by the percentage of positive birds in the unvaccinated + challenged control group x 100

†.NA- not applicable

‡. Fold change = Mean gene copy numberfrom vaccinated compared to unvaccinated group

was similar among all groups in chickens. In that respect, the present findings challenges the perceived paradigm, and adds further complexity to avian influenza vaccine virus selection, as reviewed by Spackman and Pantin-Jackwood [11]. Additional studies are warranted to strengthen that observation.

Acknowledgments

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