ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorijps@gmail.com

International Journal of Poultry Science 6 (12): 989-993, 2007
ISSN 1682-8356

Influence of Virus Strain on the Efficacy of Vaccine Against Avian Influenza Virus Subtype H7N3

S. Sattar[2], K. Naeem[1], Z. Ahmed[1] and S.A. Malik[2]
[1]National Reference Laboratory for Poultry Diseases, Animal Sciences Institute, National Agricultural Research Centre, Park Road, Islamabad, Pakistan
[2]Department of Biochemistry, Quaid-I-Azam University, Islamabad, Pakistan

Abstract: To evaluate the effect of *in ovo* passaging on the biological activity and immunogenicity of various H7N3 Avian Influenza Virus (AIV) isolates, three field H7N3 isolates (A/Chicken/Pakistan/Murree/NARC/69/04 (H7N3); A/Chicken/Pakistan/Rawalpindi/NARC/160/04 (H7N3); A/Chicken/Mansehra/NARC-214/04 (H7N3)) from the outbreak of 2003-04 were propagated *in ovo* up to 30th passage. After each 10 passages, the isolates were inactivated, injected into 10 days old chicks subcutaneously and seroconversion monitored by Haemagglutination Inhibition (HI) test. The vaccinated chicks were then challenged with the corresponding virus isolate and the mortality and virus shedding was recorded post-challenge. The results showed that the isolate A/Chicken/Murree/NARC-69/04 (H7N3) was relatively more immunogenic since the vaccinated chicks produced the highest HI antibody titers than the chicks inoculated with the other two strains of H7N3 AIV. The virus shedding upon challenge was also lowest in chickens in this group. On the contrary, the isolate A/Chicken/Mansehra/NARC-214/04 (H7N3) was less immunogenic despite repeated passaging up to 20th passage, however, the antibody titers did increase after 30th passage. The virus recovery from organs was minimal in group given vaccine prepared from Murree isolate and highest for Mansehra isolate. No mortality was observed in any of the vaccinated and challenged group of any combination in this study. This study therefore identified the Murree isolate (A/Chicken/Pakistan/Murree/NARC/69/04) as a better candidate for vaccine preparation among the currently circulating H7N3 isolates in Pakistan. The results of this study imply that a careful assessment of the vaccine potential of an isolate must be made in meeting defined vaccine strain criteria before including that isolate in vaccine production chain.

Key words: H7N3 Avian Influenza Virus, field isolates, vaccine

Introduction

Outbreaks of Highly Pathogenic Avian Influenza (HPAI) are becoming progressively more expensive to control and the economic impact on producers and consumers is a reflection of the growth of the poultry industry worldwide. It is therefore essential to reduce the amount of Avian Influenza Virus (AIV) circulating in poultry and on farms. No single set of measures is appropriate to all countries and, consequently, multiple strategies are now been used during a particular outbreak. Use of an appropriate vaccine is one of the tools which can be used effectively in the control of HPAI infection in poultry. Due to antigenic drift and antigenic shift frequently occurring point mutations in the genes coding for the two surface proteins, the Hemagglutinin (HA) and Neuraminidase (NA), allow these viruses to escape existing immunity to previously circulating influenza viruses in individual and in the population. Through this process of antigenic drift, new variants evolve and can cause epidemics in humans. In poultry such mutations in serotype H5 and H7 of AIV may result in new outbreaks of mild to severe nature. However, the rate of occurrence of antigenic drift among AIVs is usually low in nature, however, the possibilities of rapid mutations is greater in case of genetic reassortment upon infection of multiple serotypes of AIV in chickens. Use of inactivated AI vaccines is reported to reduce shedding of AIV upon new exposure, therefore, there is no way a vaccinated flock can be a greater threat to disease control than a non- vaccinated flock that breaks with AI (Karunakaran *et al*., 1987; Swayne *et al*., 1997).

An endemic HPAI virus subtype H7N3 appeared first time in northern part of Pakistan during 1994-95. The virus was apparently introduced by birds which migrated seasonally from China and Asiatic Russia (Naeem, 1998). As no funds for compensation and logistic support for mandatory culling were available from the government at that time, a decision was made to do voluntary culling and using vaccination in the affected area by homologous locally-prepared vaccine. Since the poultry population in Pakistan is largely compartmentalized in specific regions, it was possible to confine the disease to an area of 100 km in radius around the epicenter of disease. Since its first introduction in 1995 in Pakistan, two new outbreaks of AIV serotype H7N3 have been reported in the country in 2001 and 2003 (Naeem *et al*., 2006). Each time a policy of culling of the affected flocks along with strategic

vaccination using local strain from the field outbreak of AI has been employed for the control of this disease at each location. This indicates the significance of continuous virus surveillance, its typing and biological characterization for evaluating the effectiveness of the available vaccines against the newly introduced viruses at each outbreak. It has also become important to carry out simulation exercise to understand the effect of virus propagation on its mutation resulting in changed pathogenicity and/or immunogenicity. For this purpose, the study reported here was designed to *in ovo* propagate three selected isolates of AIV subtype H7N3 up to 30th passage level, to examine any change in their biological properties, especially on their immunogenic capabilities. It is anticipated that this may help in finding a better vaccine against AIV subtype H7N3 in future.

Materials and Methods

Virus: Three low path isolates of avian influenza virus subtype H7N3 were selected from the virus repository of isolates at National Reference Lab for Poultry Diseases (NRLPD), National Agricultural Research Center (NARC), Islamabad. These were: Isolate I: A/Chicken/Pakistan/Murree/NARC-69/04 (H7N3); Isolate II: A/Chicken/Pakistan/Rawalpindi/NARC-160/04 (H7N3); and Isolate III: A/Chicken/Pakistan/Mansehra/NARC-214/04 (H7N3).

Vaccine preparation: These three isolates were propagated in 9-day-old embryonated chicken eggs using the techniques of virus inoculation and harvesting as described in OIE Manual, 2004. After 10th, 20th and 30th passages, the resulting virus were used to prepare a vaccine. The virus stocks were inactivated using 0.01% formaldehyde and the inactivation confirmed using chicken embryo inoculation procedure as described by Swayne *et al.*, 1998. After inactivation, 90 mL of the inactivated virus was added to a mixture of Sorbitan mono oleate (30 mL) and mineral oil (36 mL) while blending at 2000 rpm for 5 minutes. The mixture was further blended at 8000 rpm for 5 minutes. This vaccine preparation was finally tested for sterility and safety by standard protocols described in OIE manual, 2004. In addition, the virus stocks were also tested after each passage (pre-inactivation) for HA titers as well as the EID_{50}/ml were calculated (Table 1).

Experimental animals: One-day-old broiler chicks were obtained from non AI-vaccinated parent flock and reared in isolation at the animal house at NRLPD, NARC in chicken isolators. The chickens were provided with water and feed for *ad libidum* consumption. The selected chickens were later on shifted to chicken isolators for challenging with live AIV subtype H7N3.

Experimental design and endpoints: A total of 40 day old chicks obtained from non-AI vaccinated parent chickens were divided into two groups. The chicks in Group A (n = 30) were given single vaccine dose whereas the chicks in Group B (n = 10) served as unvaccinated control. The chicks in Group A were further sub-divided into three sub-groups (a-c); one for each of the three passages (10th, 20th and 30th passage) of each isolate. This design was repeated for each of the three H7N3 isolates. Each chick was given 0.3 mL of vaccine sub-cutaneously at 10 day of age. The chicks were bled weekly for six weeks and sero-conversion was monitored by HI test. After six weeks post-vaccination (PV) three chickens from group A and two from group B were challenged with AIV H7N3 intra-nasally using a dose of $10^{7.5}$/0.1 mL EID_{50} titer. Clinical responses were monitored for 12 days post-challenge (PC). Shedding and infection was checked by taking cloacal and tracheal swabs every 3rd day PC from chicks in each group and processed for virus isolation as per standard protocols. Chickens were bled at every 3rd day PC to detect efficacy of different vaccines at various passage level from each group. After 12 days of PC, the chickens were euthanized and trachea, lungs, intestine and pancreas were collected for AIV detection using IFA and egg inoculation.

Evaluation of seroconversion against H7N3 was carried out by Haemagglutination Inhibition (HI) test following the procedure described by Beard (1989).

The cloacal swabs were collected from experimental chicks at each 3 days interval Post Challenge (PC) and placed in 1.5 mL of Brain Heart Infusion (BHI) broth, containing antibiotics (Gentamycin 100 mg/ml, Amphoteracin-B 5 mg/ml and Penicillin 150 mg/ml).

Isolation and identification of viruses: The organs collected from the challenged group of chickens were subjected to virus isolation by *in ovo* inoculation following the protocol as described in OIE Manual, 2004.

Indirect Immunoflouresent Assay (IFA): The indirect immunoflourecence assay was performed on tissue specimens collected from the chickens surviving the challenge of AIV. In this regard a 4 mm^3 organ piece was embedded in OCT Compound (Miles, Inc., USA) using plastic troughs and freezing at-20°C. This was subjected to cryo-sectioning to 4 micron size using microtome (Miles, USA). Appropriate dilution of hyper-immune serum against AIV (H7N3) was employed followed by FITC labeled anti species IgG antibodies (Sigma USA) using protocol described by McFerran (1980). The presence of AIV antigen in the tissues was ascertained by the presence of antigen-specific nuclear and cytoplasmic fluorescence microscopically.

Results

The pre-inactivation HA titers and EID_{50} values corresponding to the three isolates and their relative passage levels are shown in Table 1. In general the HA

Table 1: Characteristics of Avian Influenza H7N3 Isolates used in this study

Isolate	Designation	Passage level (P)[1]	Pre- inactivation HA titers	Pre-inactivation EID_{50}/mL
I	A/Chicken/Pakistan/Muree/NARC-69/04(H7N3)	a-P10	512	8.5
		b-P20	256	8.5
		c-P30	2048	8.5
II	A/Chicken/ Pakistan /Rawalpindi /NARC-160/04 (H7N3)	a-P10	512	8.5
		b-P20	4096	8.5
		c-P30	2048	8.5
III	A/Chicken/Pakistan/Mansehra/NARC-214/04(H7N3).	a-P10	4096	7.23
		b-P20	256	8.5
		c-P30	4096	9.5

[1]a, b, c indicates three groups of chicks inoculated with H7N3 isolate at 10, 20 or 30th passage level

Table 2: Immune Response of Chicks after Exposure to Vaccine prepared against three H7N3 Avian Influenza Isolates and post-challenge at the Indicated Passage Levels

		Haemagglutination Inhibition (HI) Antibody Response[2]						
		Weeks Post Vaccination (WPV)						HI titers (GMT, Log_2)
	Passage							Post-challenge (PC)[3]
Isolate[1]	No.	1	2	3	4	5	6	Day 12 PC
Geometric mean titers (Log_2)								
Isolate I	10th	3.4	4	5.6	5.5	5.8	5.9	9
	20th	0.75	4.63	8.63	7.88	7.88	7.63	6.8
	30th	3.13	3.5	6.85	6.14	7.5	6.13	6.8
Isolate II	10th	2.25	4	4.88	5.63	5.25	5.75	6.6
	20th	3.75	2.17	6	6.14	4.75	4	3.2
	30th	3.13	4	8	7.5	7	6.4	6.8
Isolate III	10th	1.88	2.4	5.6	6.8	4.78	3.78	9.25
	20th	0.63	0.87	1.4	2.2	2.5	2.75	2.8
	30th	2.5	3.8	4.6	6.5	6.9	7.5	3.88
Unvaccinated control	-	0	0	0	0	0	0	9.0[4]

[1]Isolate designations are provided in Table 1. [2]Ten days old chicks were inoculated with 0.3 mL of inactivated vaccine prepared for each of the three H7N3 isolates (n = 10 chicks/isolate). [3]Vaccinated chicks were challenged with $10^{7.5}$/0.1 mL EID_{50} and antibody titres determined as indicated. [4]Represents antibody titres of un-vaccinated control chicks which survived the H7N3 challenge

titers, as an indicator of the amount of virus, were higher with the increased passage level but with the exception of isolate III. The EID_{50}/mL was quite comparable between all isolates at various passage levels (Table 1).

Table 2 shows the seroconversion potential over a six weeks period of various isolates at three different passage levels after a single injection with the inactivated (i.e., vaccine) virus inoculum. The data indicates that the isolate I had a numerically higher seroconversion (i.e., five time points with GMT values of 7 or greater) than the rest of the two isolates. The isolate II was intermediate in seroconversion response where as the isolate III had numerically a lowest response. The antibody titers were also determined of immunized chicks at 12 days post challenge with the corresponding three isolates. The data shows that the isolate I chicks responded better with relatively higher GMTs as compared with the chicks in isolate II and III groups, suggesting that the isolate I was better in boosting the previously immunized chicks.

Table 3 describes the data from the challenge experiments in which the mortality, virus presence and/or shedding was used as an endpoint. No mortality was observed in any of the immunized and challenged chick groups. However, 9 out of 15 chicks in unvaccinated groups died after the challenge. Virus isolation from tissues studies indicated that isolate III was more readily propagated and isolated after egg inoculations than the rest of the two isolates. Virus shedding was interestingly lowest in challenged chicks which were immunized with the isolate III vaccine. The isolation studies from various organs at post vaccination and post challenge reveal that lungs and trachea are the most prevalent sites for the virus re-isolation (Fig. 1).

Discussion

Avian influenza vaccines against low pathogenic serotypes of H5, H7 and H9 have been successfully employed in several countries for its control (Halvorson, 1998; Daprile, 1986). Outbreaks of highly pathogenic serotypes of H7 and H5 are traditionally controlled by programmed eradication. However successful use of vaccines in controlling HPAI outbreaks in chickens has been reported in the recent past (Naeem, 1998: Garcia *et al.*, 1998).

Influenza is a dynamic disease owing to the polymorphic character of influenza virus. Due to antigenic drift influenza viruses frequently accumulate point mutations

Table 3:Post-challenge mortality and recovery of AIV subtype H7N3 from different organs of experimentally vaccinated and challenged chickens

Vaccine ID	Passage level (P)	Group ID	Mortality 12th day PC Dead/ Total challenged	Organ Positive for Virus Isolation up to 12 day PC				Shedding pattern 12th day PC	
				Trachea	Intestine	Lung	Pancreas	Cloacal Recovery	Oropharyngeal/ Tracheal Isolation
Isolate I	10	a-10-P	0/5	-	-	-	-		
	20	b-20-P	0/5	-	-	-	-		
	30	c-30-P	0/5	+°	-	-	+°	++°	
Isolate II	10	a-10-P	0/5	-	-	-	-		+°
	20	b-20-P	0/5	-	-	+°	-		
	30	c-30-P	0/5	+•	-	+•	-	++°	++°
Isolate III	10	a-10-P	0/5	+°	-	-	+°		
	20	b-20-P	0/5	-	+°	-	-		
	30	c-30-P	0/5	-	-	+°	-		
Group B	Control		9/15	+°	+°	+°	+°	++°	++°

+°= Positive by IFA and egg inoculation, +• = Positive by IFA only

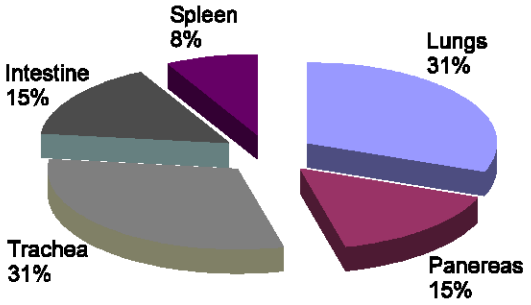

Fig. 1: Distribution of AIV subtype H7N3 amongst various organs from experimentally challenged chickens as determined by Egg inoculation and Immuno Florescence Assay (IFA)

in the hemagglutinin and neuraminidase surface protein genes generating new variants capable of escaping existing immunity. Therefore, a need arises to periodically replace vaccine strain in relation to circulating field virus population. Moreover vaccines having good antigenic match with challenge virus have repeatedly given best protection following exposure (Swayne *et al.*, 1999).

Present study was designed in a similar perspective to select appropriate vaccine strain of AIV H7N3 from the circulating strains of AIV in Pakistan which is more immunogenic and resistant to challenge with field isolates. Vaccines were prepared from three AIV H7N3 field isolates after propagating them at 10th 20th and 30th passage levels in embryonated eggs. Although the tested strains differed in inducing antibody response, all of them exhibited uniform protection against clinical signs and mortality following challenge. In a similar study by Swayne *et al.* (1999) different isolates of AIV H5 were used to develop inactivated vaccines, followed by challenge with field isolates. All the vaccines provided good protection from clinical signs and death and produced positive serological reactions.

The potency of vaccine is basically evaluated by testing the level of immune response, reduction in virus shedding upon field exposure of the virus and protection from clinical signs and death upon field challenge (Stone, 1987; Swayne and Stone, 1996). The test vaccines were subjected to these criteria in the study. The results indicated that all the vaccines qualified the above criteria, indicating cross protection of different strains used in the vaccine. However, oropharyngeal isolation of challenge virus was regularly observed among the vaccinated birds.

The comparison of various isolates in terms of inducing an immunogenic response clearly shows that isolate I and isolate II were better immunogenic as compared with the isolate III. While isolate III was less immunogenic at 10th and 20th passage level vaccines leading to the production of low levels of antibody titers, it exhibited a drastic improvement in GMT titer in 30th passage vaccinates (Table 3), indicating a distinct effect of passaging on immunologic properties of the virus. Antibody titers in these vaccinates were not persistent since they gradually depleted by 6th week post vaccination, after peaking at 4th week post-vaccination. This indicated a change in immunological properties of the isolate III by repeated *in ovo* passaging. Similar results were reported by Horimoto and Kawaoka (1995). This improved immunogenicity after *in ovo* passages may be the reason why the isolate III vaccinated and challenged chicks showed the least amount of virus shedding as compared to the rest of the chick groups. Interestingly, all vaccinated groups survived the challenge. It would be interesting to know if the survival rate would have been the same if a higher concentration of challenge virus would have been used. In this study we only used one tenth of the EID_{50} dose for challenge which may not be very representative of the natural exposure in the field.

The criteria for selection of a virus strain for use in commercial vaccine preparation include the higher rate of replication in embryonated eggs, reduced viral shedding upon infection in vaccinated chickens,

prolonged antibody titers and poor replication of challenged virus in the vaccinates (Swayne and Stone, 1996). Based on the above criteria, this study shows that isolate A/Chicken/Murree/Pakistan/NARC-69/04 (isolate I) fulfils the criteria for use as vaccine, as it propagated efficiently in embryonated eggs giving rise to high HA titers, produced prolonged immunity when used in the vaccine, hindered virus shedding upon challenge and its immunogenic capabilities were not affected by repeated *in ovo* passaging. It also confirmed that close antigenic compatibility between vaccine strain and challenge virus gives best result as earlier suggested by Swayne *et al.* (1999). It is therefore, high time that the above referred local strains of AIV are included in the Avian Influenza killed vaccines produced within the country.

References

Beard, C.W., 1989. Serological procedures. In: A laboratory manual for the isolation and identification of avian pathogens. 3rd Ed. American Association of Avian Pathologists, Kennett Square, PA. USA, pp: 192-200.

Daprile, P.N., 1986. Current situation of avian influenza in Italy and approaches to its control. In: Acute virus infection of poultry, McFerran J.B. &McNulty M.S., eds. Martinus Nijhoff, Dordrecht, The Netherlands, 29-35.

Garcia, A.J., D.K. Srivastava, D.A. Jayawardene, D.R. Wehr and R.G. Webster, 1998. Efficacy of inactivated H5N2 influenza vaccines against lethal A/chicken/Queretaro/19/95 infection. Avian Dis., 42: 248-256.

Halvorson, D.A., 1998. Strengths and weaknesses of vaccines as a control tool. Proceedings of the Fourth International Symposium on Avian Influenza (pp: 223-227). United States Animal Health Association.

Horimoto, T. and T. Kawaoka, 1995. Molecular changes in virulent mutants arising from avirulent Avian Influenza during replication in 14-day old embryonated eggs. Virol., 206: 755-759.

McFerran, J.B., 1980. Avian Adenoviruses. In: A laboratory manual for the isolation and identification of Avian Pathogens, 2nd Edition. American Association of Avian Pathologists, American Association of Avian Pathologists, Kennett Square, PA. USA.

Karunakaran, D., J.A. Newman, D.A. Halvorson and A. Abraham, 1987. Evaluation of inactivated influenza vaccines in market turkeys. Avian Dis., 31: 498-503.

Naeem, K., 1998. The avian influenza H7N3 outbreak in south central Asia. In: D.E. Swayne and R.D. Selmons (Eds). Proc. 4th. Int. Symp. on avian influenza. Pages 31-35, American Association of Avian Pathologists.

Naeem, K., N. Siddique, M. Ayaz and M.A. Jalali, 2006. Update of Avian Influenza in Pakistan. Avian Dis., 51: 189-193.

Office International des Epizooties (OIE), 2004. OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Paris: Office International des Epizooties, pp: 2.2.14.

Stone, H.D., 1987. Efficacy of avian influenza oil-emulsion vaccines in chicken of various ages. Avian Dis., 31: 483-490.

Swayne, D.E., J.R. Beck, M. Garcia and H.D. Stone, 1999. Influence of virus strain and antigen mass on efficacy of H5 avian influenza inactivated vaccines. Avian Pathol., 28: 245-255.

Swayne, D.E., J.R. Beck, M.L. Perdue and C.W. Beard, 2001. Efficacy of vaccines in chicken against highly pathogenic Hong Kong H5N2 avian influenza. Avian Dis., 45: 355-365.

Swayne, D.E. and H.D. Stone, 1996. Evaluation of inactivated H5 avian influenza virus and fowl pox recombinant vaccines and diagnostic test reagents; implication in avian influenza control and prevention strategy. In: Proc. 45th Western Poultry Disease Conference, May 1996, Cancun, Mexico, pp: 35-38.

Swayne, D.E., J.R. Beck and T.R. Mickle, 1997. Efficacy of recombinant fowl pox vaccine in protecting chicken against highly pathogenic Mexican origin H5N2 avian influenza virus. Avian Dis., 41: 910-922.

Swayne, D.E., M.L. Perdue, N. Cox, T. Rowe, C. Bender, J. Haung and D.L. Saurez, 1998. Comparisons of highly virulent H5N1 Influenza A viruses isolated from humans and chickens from Hong kong. J. Viro., 72: 6678-6688.