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Effect of Induced Mutation on Pathogenicity Index of Avian Influenza Virus Subtype H7N3

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Abstract: Intravenous pathogenicity index (IVPI) of three Avian influenza virus (AIV) isolates of serotypes H7N3 was determined at their zero, 10 ,20 and 30th *in ovo*-passage levels to check the effect of mutation during passage on pathogenicity of the virus. For this purpose three local field isolates of AIV H7N3 were passaged in 9 day old chicken embryonated eggs. Each passaged isolate was subjected to biological evaluation at every 10th passage level. It was found that *in-ovo* passaging of viruses had both positive and negative effect on pathogenicity index of H7N3 isolates. It was also determined that among three isolates only one isolate showed increase in pathogenicity by passaging while the other two showed decline in their pathogenicity index. On the other hand repeat passaging helped to increase HA titers in all the isolates.

Key words: Avian Influenza, serotype H7N3, induced mutation

INTRODUCTION

Emergence of Highly Pathogenic Avian Influenza (HPAI) in Asia is an ongoing economic threat along with its potential as public health problem. Avian influenza (AI) is a clinically variable epizootic disease caused by antigenically and pathogenically diverse type A influenza viruses. Influenza viruses have a worldwide distribution and although not endemic in commercial poultry, sporadic outbreaks of subtype H5 and H7 do occur. In recent times these outbreaks have been occurring with increasing regularity. Outbreaks are typically of a Low Pathogenic form of Avian Influenza (LPAI). It is assumed that in a susceptible domestic poultry population, circulating LPAI (especially H5 and H7) has the ability to mutate into a more devastating highly pathogenic avian influenza (HPAI) resulting in Fowl Plague.

Influenza viruses exist as diverse serotypes in birds, particularly in wild waterfowl (Suarez *et al*., 2003). All the available evidence suggests that the most common primary introduction of AI viruses into an area is by wild birds, usually waterfowl, as it is known that wild waterfowl present a natural reservoir for these viruses, but gulls and shore birds have also been implicated (Kawaoka *et al*., 1988). Avian influenza in Pakistan was first documented in 1995. The disease caused by H7N3 affected mainly a population of 32 million birds primarily breeders and broilers of cold northern region of the country. The outbreak resulted into an estimated economic loss of 814.4 million rupees (Naeem and Hussain, 1995). Subsequent outbreaks were reported in 1998 and 2003 in different parts of the country by Naeem *et al*. (1999) and Iqbal *et al*. (2003).

The pathogenicity of AI viruses is extremely variable which cannot be predicted due to its specificity to the type of bird and its original host, as true with AIV subtypes H1-H3 (human influenza viruses). As practiced in human influenza virus control program, there is a need to adjust the tendency of influenza viruses to undergo frequent and permanent antigenic changes, thereby necessitating a constant monitoring of the global influenza situation and annual adjustments in the composition of human influenza vaccines. Due to tendency of human influenza viruses (H1, H2 and H3) to undergo frequent antigenic changes, the composition of human influenza vaccines is annually adjusted. Similarly, it is predicted that animal influenza vaccines may require changes due to its mutations during field passages. It therefore, is important to mimic the field mutation among influenza viruses, in the lab. Study reported here was designed to compare pathogenicity of various high path and low path H7N3 isolates after *in ovo* passaging as an indication of induce mutation in HPAI subtype H7N3 and to see its effect on various biological properties of the isolates. It is hypothesized that through induced mutation in field isolates of HPAI subtype H7N3, there is a possibility of selecting some LPAI strains, which may serve as better vaccine candidate against HPAI viruses in future.

MATERIALS AND METHODS

Virus: The following selected isolates of AIV subtype H7N3 isolates were obtained from the repository of viruses at the National Reference Lab for Poultry Diseases (NRLPD), Animal Sciences Institute, National Agricultural Research Centre (NARC), Islamabad, Pakistan:

- Isolate K-03: A/Chicken/Karachi/Pakistan/NARC-23/2003 (H7N3).

- Isolate S-03: A/Chicken/Sheikhupura/Pakistan/NARC-43/2003 (H7N3).
- Isolate M-04: A/Chicken/Murree/Pakistan/NARC-517/2004 (H7N3).

Virus passaging: The selected isolates were passaged in 9-day old chicken embryonated eggs via allantoic inoculation up to 30th passage level by using the techniques of virus inoculation and harvesting as described in OIE Manual (OIE Manual, 2004). In addition, the virus stocks were also tested after each passage for HA titers as well as for EID_{50}/mL by using Reed and Muench method (Reed and Muench, 1938) (Table 1). Only one isolate was tested at a time to avoid any cross contamination.

Experimental animals: In order to determine the Intravenous Pathogenicity Index (IVPI), commercial broiler chicks were purchased from non-AI vaccinated parent flock at one day of age. They were reared in chicken isolators at the animal house facility at NRLPD, NARC. The chickens were provided with water and feed *ad libidum*. The AIV subtype H7N3 challenge studies were conducted on selected chickens at four weeks of age.

Experimental design: A total of 130 day old broiler chicks obtained from non-AI vaccinated parent chickens were divided into 4 different subgroups per virus isolate for determining the IVPI at 0, 10, 20 and 30th passage level (Table 2). A dose of $10^{7.5}$/0.1 mL EID_{50} titer of passage virus isolate was inoculated intravenously to each chicken. One control group of un-inoculated birds was reared in a separate chicken isolator. Every third day Post Inoculation (PI), these birds were swabbed via cloaca and tracheal route. After 7-days PI the remaining birds were bled for serological evaluation. Trachea, heart, spleen, intestine, pancreas, lungs and thymus were collected from birds died during challenge for subsequent detection of AIV by Immuno-Fluorescence Assay (IFA) and egg inoculation method. The mortality was recorded to work out IVPI of each isolate accordingly.

Evaluation of sero-conversion 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 every 3 days post challenge (PC) and placed in 1.5 mL of Brain Heart Infusion broth (BHI), 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 (OIE Manual, 2004).

Table 1: Comparative evaluation of biological activities of the H7N3 AIV isolates

ID of AIV Isolates	Passage levels	HA titres (log_2) of passaged isolate	EID_{50} (log_{10}) in chicken embryonated eggs	IVPI (Value)
(K-03)	0	9	5.5	0.48
	10	11	7.2	1.04
	20	10	7.7	0.92
	30	13	7.8	1.22
(S-03)	0	3	4.5	2.20
	10	12	8.5	2.31
	20	13	9.3	2.25
	30	12	9.2	1.83
(M-04)	0	6	7.2	2.30
	10	10	8.5	2.24
	20	12	8.5	2.02
	30	13	9.2	2.14

Indirect Immunoflorecent Assay (IFA): The indirect immuno-fluorescence 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 freezed 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 subtype 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

Table 2 shows the comparative HA titers, EID_{50} and IVPI values of the three H7N3 AIV isolates. The data indicate an increase in HA titer with the corresponding increase with the virus passage level for all three isolates tested. While the baseline HA activity was variable at 0 passage level, the increase in HA titer (representing virus concentration) was comparable between all virus isolates. The EID_{50} increased over passage time as expected for each virus isolate. IVPI scores for all isolates ranged from 0.48-2.31. Isolate K-03 exhibited an increase in pathogenicity over passage time as evident by increase from 0.48-1.22 IVPI value. However, the isolates S-03 and M-04 did not show any significant change over passage time. Antibody titers against isolates and virus shedding post inoculation data are provided in Table 3. Chicks inoculated with K-03 isolate sero-converted well at 7 DPI with a GMT range of 5.8-8.5 at various passage levels. For S-03 and M-04 isolates no HI titers could be determined due to early mortality in all subgroups except 10th passage group of isolate S-03 in which the survived chicks had a GMT of 1.7. Virus shedding for K-03 isolate was positive for 3, 6 and 9 DPI. On the contrary, no virus shedding was observed

Table 2: Experimental design[1]

Chicks	ID of Avian Influenza Virus H7N3 isolate used	Group ID with passage level of AIV used: Sub-group (n = 10/group)	Passage level
Group A (n = 40)	K-03 (A/Chicken/Karachi/Pakistan /NARC- 23/2003 (H7N3)	A-1	0
		A-2	10th
		A-3	20th
		A-4	30th
Group B (n = 40)	S-03 (A/Chicken/Sheikhupura /Pakistan /NARC-43/2003 (H7N3)	B-1	0
		B-2	10th
		B-3	20th
		B-4	30th
Group C (n = 40)	M-04 (A/Chicken/Murree/Pakistan /NARC-517/2004 (H7N3)	C-1	0
		C-2	10th
		C-3	20th
		C-4	30th
Group D (n = 10)	Un-Inoculated (control group)	D-1	Un-Inoculated

[1]The control and intravenously virus inoculated chicks were sampled for Cloacal swabs for virus isolation at 3, 6 and 9 days and bled for antibody titers at 7 day post inoculation

Table 3: Post-Inoculation characterization of AIV isolates of subtype H7N3 at different passage levels

ID of AIV Isolates	Passage level	Antibody titers at 7th Day PI: No. of birds tested	AIV H7 HI antibody titers (GMT log_2)	Detection of Virus Shedding at different days PI: 3rd day	6th day	9th day
K-03	0	10	5.8	+	+	+
	10th	10	6.1	+	+	+
	20th	10	5.8	+	+	+
	30th	10	8.5	+	+	+
S-03	0	10	ND*	+	-	ND*
	10th	10	1.7	+	-	ND*
	20th	10	ND*	+	-	ND*
	30th	10	ND*	+	-	ND*
M-04	0	10	ND*	+	ND*	ND*
	10th	10	ND*	+	-	ND*
	20th	10	ND*	+	-	ND*
	30th	10	ND*	+	ND*	ND*
Control	Non-Inoculated	10	0.0	-	-	-

ND* = Not done due to early mortality of chickens, PI = Post Inoculation

Table 4: Post Inoculation distribution pattern of AIV Isolates (H7N3) in visceral organs

ID of AIV Isolates	Passage levels	Virus isolation post infection: Lungs	Trachea	Heart	Kidneys	Spleen	Intestines	Thymus	Pancreas
K-03	0	-	+	-	+	-	-	-	+
	10	+	+	+	+	+	+	+	+
	20	+	+	+	+	+	+	+	+
	30	+	+	+	+	-	-	-	+
S-03	0	+	-	+	+	+	-	-	+
	10	+	-	+	+	+	-	-	+
	20	+	-	+	+	-	+	+	+
	30	+	+	+	+	+	+	+	+
M-04	0	-	-	+	+	+	+	-	+
	10	+	+	-	+	+	+	+	+
	20	+	+	+	+	+	+	+	+
	30	+	-	-	+	-	-	-	+

Note: All the organs of control bird showed negative results

at 6 DPI for isolate S-03. Also, no virus shedding was observed at 10 and 20th passage group for M-04 isolate at 6th DPI. No virus isolation study was possible for isolates S-03 and M-04 beyond 6th DPI because of mortality. Virus neutralization test was carried out on only those samples that showed positive HA activity upon egg inoculation. All the positive samples turned out to be AIV subtype H7N3. All Virus isolates were easily isolated from kidneys, pancreas and lungs where as isolation from trachea, heart, spleen, intestine and thymus was

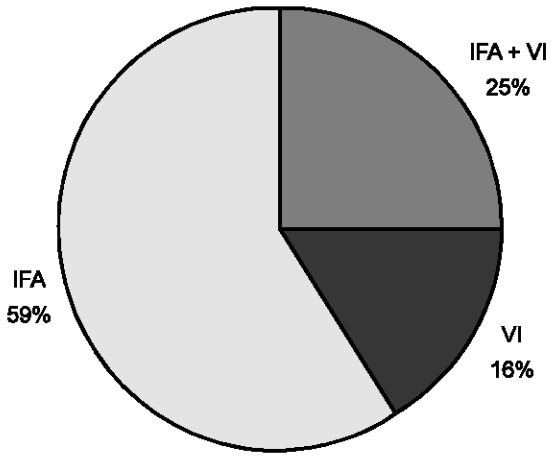

Fig. 1: Comparison of different diagnostic techniques for AIV diagnosis. VI: Virus Inoculation, IFA: Immuno Fluorescent Assay

at a lesser and variable degree (Table 4). The IFA technique was found to be more sensitive for detection of all three viral isolates in the tissues. A 59% of the total tissue samples tested were IFA positive for various H7N3 AIV isolates, whereas 25 % of the samples were found positive by both IFA and virus inoculation techniques. Only 16% samples were detected positive using virus inoculation technique (Fig. 1).

DISCUSSION

It is commonly understood that all the Avian Influenza (AI) viruses undergo the processes of mutation during circulation in susceptible birds. It is also known that the pathogenicity of AI viruses is extremely variable and cannot be predicted in the host of origin. For example, the AI subtypes H5 and H7 have been associated with severe disease outbreaks in chicken and turkeys but many AIV subtypes of H5 and H7 isolates are also known to be either non-pathogenic or only moderately pathogenic (Beard and Easterday, 1973; Easterday *et al*., 1997). Thus, it is important to determine if a particular AIV isolate tends to change its biological behaviors upon *in ovo* passaging which could possibly convert a high path isolate of a virus into a low path and vice versa.

The present study was an attempt to examine any changes in AIV virulence following various *in-ovo* passages. The AIV isolate K-03 used in this study will be considered as low path, as it showed 0.48 IVPI score. There was no mortality, clinical signs and gross lesions seen on the organs of affected birds from which, it was isolated. When virus was passaged *in ovo* up to 10th passage level, its IVPI index changed to 1.04, declining to 0.92 at 20th passage and again increased to 1.22 at 30th passage. This indicates that the viruses may revert to high path, if more passaging is continued. This mutation may have occurred at HA genome, as it has been earlier reported that replacement of a few amino acids, at HA cleavage, because of gene mutation can convert a low path AIV into high path (Wood *et al*., 1993). However, it would be worth carrying out sequences analysis of this passaged virus to specifically identify the mutated region at HA genome.

AIV isolate K-03 although showed high HA titre through out thirty passages, its EID_{50} values gave a different pattern. Its EID_{50} at zero passage was $10^{5.5}$ which seem to be increased due to the effect of passaging, as at 10th passage it gave $10^{7.2}$ EID_{50} value. At 30th passage this value increased to $10^{7.8}$, which shows mutation may have occurred during passaging. However, it was noticed here that IVPI values of all the isolates, did not change significantly in the passaged virus.

As this virus was recovered from an earlier AI outbreak of low intensity in Karachi in April 2003, the same virus appears to have mutated in the field and resulted into high path AIV in Oct 2003, causing heavy mortality in the field. Similar information was reported from Italy during Italian outbreak of $H7N_1$ in 1999-2000.It is believed that a LPAI AI virus of subtype $H7N_1$ converted into HPAI (Marangon *et al*., 2003). In another report in 2004 a Highly Pathogenic Avian Influenza (HPAI) outbreak erupted in British Columbia. Investigations indicated that the responsible isolate of HPAI H7N3 emerged suddenly from low pathogenic precursor, previously known to persist in the area (Pasick *et al*., 2005).

The immunogenicity studies of these isolates indicate that the low path virus (K-03) showed high antibody titers as compared to other two isolates. Upon inoculation with zero-passaged isolate of this virus, HI antibody titer of 5.8 was recorded. This later on increased to 8.5 when the virus from 30th passage was inoculated in susceptible chickens. It indicates that repeated *in ovo* passaging of a LPAI may even help to select a more immunogenic strain of AIV irrespective of changing its pathogenicity. This passaged virus may serve as a vaccine candidate in future, once potency evaluation for vaccines prepared from this isolate are also carried out.

It has been earlier reported that birds that survive from the infection of AIV H5N1, excrete virus for at least 10 days, orally and in feces, thus facilitating further spread at live poultry markets or transferring via wild and/or migratory birds (Webster *et al*., 1978). The present study showed similar results with H7N3 isolates, as the low path isolate (K-03) excreted the virus up to 10 days post exposure. All the passages of this isolate presented the same shedding pattern. The K-03 was LPAI so it had made the virus shedding possible for a longer period of time (Table 3).The low path viruses are able to live in their host for longer time and are also able to successfully transfer to other healthy birds of flocks. So its chance of mutation is increased many fold. This

further supports our earlier assumptions that the low path of AIV H7N4 2003 may have mutated to high path through circulation in commercial flocks and eventually resulted in the high path outbreak of Oct 2003 in Karachi. The clinical signs produced upon viral exposure also varied between different passage levels of K-03. No mortality was observed in chickens exposed to zero passaged AIV H7N3. However, upon the exposure of chickens to 10 and 20th passage level of this virus, birds showed mild clinical signs and mortality were also observed. Although, these clinical signs were not pronounced but it does indicate some variation might have occurred in viral pathogenicity from 0-30 passage levels. At the level of 30th passage when virus became mildly pathogenic, it showed more pronounced signs and symptoms of the AI disease, such as fever, dizziness, diarrhea, facial edema and nasal secretion. Our results are similar to the previous findings of Swayne and Halvorson (2003) who showed that clinical signs of LPAIV primarily confine to mild respiratory disease, depression and drop in egg production in laying birds.

The Sheikhupura isolate (S-04) showed extremely high variation in its EID_{50} values. Usually HPAI emerges from their LPAI counterparts, but isolate from Sheikhupura when subjected to 30 passages, its ability to develop clinical sings declined nearing last passage. The IVPI score at zero, 10, 20 and 30th passage was 2.20, 2.31, 2.25 and 1.83, respectively. Although with this score the isolate still has to be typed as high path but in comparison with 20th passage data this IVPI index value reflects a slight decline. The birds in this group showed milder gross lesions, but they died after a prolonged clinical course, resulting in distinct peticheal hemorrhages on legs. The carcase appeared dehydrated, with cyanotic wattles.

The Murree-isolate (M-04) also showed high HA titers through out its passaging and its EID_{50} values remained higher than Karachi isolate, through out the experiment. The IVPI index of this isolate at zero, 10, 20 and 30th passage provided clear evidence that this isolate was of high path type and remained stable in its biological characteristics. It appears that passaging even at 30th level did not induce any mutation in this isolate.

In the present study, Muree and Sheikhupura isolates continuously behaved like HPAI even after 30th passages and were re-isolated from all the vital organs after *in ovo* passaging. Despite some effect on certain specific biological characteristics during *in ovo* passaging, no major mutation appears to have occurred in HA region of these isolates. This also reiterates the earlier known principle that point mutations are very slow and rare in AIV H7 subtypes in nature. It may therefore, require some other factors along with passaging of HPAI to revert it to LPAI at lab level.

Although, the IVPI test is employed to detect biological differences among the viruses, we are concerned that using illness and death as test criteria will limit the scope of biological differences that can be detected among low-pathogenic and nonpathogenic type A influenza viruses. Also, the IVPI test does not provide insight into which AI viruses may be prone to infectivity enhancement by the presence of other factors. Therefore, experiments incorporating more extensive virus isolation attempts from extra respiratory and extra-enteric tissues and histopathologic assessments to detect microscopic lesions would undoubtedly be required to provide more detailed data for identifying differences in the pathogenicity potential of passaged viruses of HPAI.

The sensitivity of IFA used in this study is reflected from the fact that 59% samples were positive by IFA rather than Virus Isolation (VI) which showed 16% samples positive. It means that some virus inoculations were either negative originally or the virus was lost during sample processing. The livability of virus becomes questionable if samples are not preserved or transported properly. IFA is a cheap and less time consuming procedure for detection of AI viral antigen. So, where appropriate, it can be used for routine disease diagnosis of AIV. The use of IFA as diagnostic tool where VI facilities to handle virus isolation of HP AIV is difficult to arrange has also been suggested by Zhou *et al*. (1999).

From this study 2 main conclusions can be drawn. Firstly, the AIV isolates are biologically different not from each other but also between the same isolates after some passages in lab or in nature. Passaging in 9-day old embryonated chicken egg has both positive and negative effect on pathogenicity index of H7N3 isolates, which can be exploited for selecting strains for H7 vaccines. It also points out the need for continuous change in vaccine strains of AIV, is also required in line with the emergence of new pathogenic strains of AIV in the field. Secondly, IVPI alone does not predict pathogenicity index of a strain. The properties of sero-conversion, shedding pattern and tissue dissemination pattern can also be helpful to define the pathogenic potential of a virus and can help in selection of an isolate for use in vaccine production.

In this scenario, the presence of high path avian influenza virus in poultry in this country and in other countries in the region poses a continuous threat for the emergence of more pathogenic strains of both avian and human influenza viruses. For this purpose there is a constant need to carry out a coordinated surveillance for influenza viruses both in birds and humans and their further characterization in all parts of the world. Better understanding of the AI viruses can help in devising appropriate control measures against the infections of HPAI both in poultry and humans, including development of an efficacious vaccine.

REFERENCES

Beard, C.W. and B.C. Easterday, 1973. A turkey/ Oregan/71 an avirulent influenza isolate with hemagglutinin of plague virus. Avian Dis., 17: 173-181.

Beard, C.W., 1989. Serological procedures. In: A Laboratory Manual for the Isolation and Identification of Avian Pathogens. 3rd Edn. American Association of Avian Pathologists, Kennett Square, PA. USA, pp: 192-200.

Easterday, B.C., V.S. Hinshaw and D.A. Halvorson, 1997. Influenza In: Calnek, B.W., H.J. Barnes, C.W. Beard, L.R. McDougald and Y.M. Saif (Eds.). Diseases of poultry. 10th Edn. Lowa State University Press, Ames, pp: 583-605.

Iqbal, M., R. Manzoor and A.W. Qazi, 2003. Isolation and identification of avian influenza virus in layer flock at Karachi-Pakistan. Pak. J. Vet. Res., 1: 37-39.

Kawaoka, Y., T.M. Chambers, W.L. Sladen and R.G. Webster, 1988. Is the gene pool of influenza viruses in shorebirds and gulls different from that in wild ducks? Virol., 163: 247-250.

Marangon, S., L. Bortolotti, I. Capua, M. Bettio and M. Dalla Pozza, 2003. Low-Pathogenicity Avian Influenza (LPAI) in Italy (2000-01): Epidemiology and control. Avian Dis., 47 (3 Suppl): 1006-1009.

McFerran, J.B., 1980. Avian Adenoviruses. In: A Laboratory Manual for the Isolation and Identification of Avian Pathogens. 2nd Edn. American Association of Avian Pathologists, American Association of Avian Pathologists, Kennett Square, PA. USA., 72: 6678-6688.

Naeem, K. and M. Hussain, 1995. An outbreak of avian influenza in poultry in Pakistan. Vet. Rec., 137: 439.

Naeem, K., A. Ullah, R.J. Manvell and D.J. Alexander, 1999. Avian influenza a subtype H9N2 in poultry in Pakistan. Vet. Rec., pp: 145-560.

Office International des Epizooties (OIE), 2004. OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Paris: Office International des Epizooties, pp: 2:14.

Pasick, J., K. Handel, J. Robinson, J. Copps, D. Ridd, K. Hills, H. Kehler, C. Cottam-Birt, J. Neufeld, Y. Berhane and S. Czub, 2005. Intersegmental recombination between the haemagglutinin and matrix genes was responsible for the emergence of a highly pathogenic H7N3 avian influenza virus in British Columbia. Gen. Virol., 86: 727-731.

Reed, L.J. and H. Muench, 1938. A simple Method of Estimating 50% end points. Am. J. Hyg., 27: 493-497.

Suarez, D.L., E.Spackman and D.A.Senne, 2003. Update on molecular Epidemiology of H1,H5 and H7 influenza virus infections in poultry in North America. Avian Dis., 47: 888-897.

Swayne, D.E. and D.A. Halvorson, 2003. Influenza. In Diseases of Poultry. In: Saif, Y.M. (Ed). Ames, IA, Iowa State Press. Blackwell Publishing Co), pp: 135-160.

Webster, R.G., M. Yakhno, V.S. Hinshaw, W.J. Bean and K.G. Murti, 1978. Intestinal influenza: replication and characterization of influenza viruses in ducks. Virology, 84: 268-278.

Wood, G.W., J.W. McCauley, J.B. Bashirruddin and D.J. Alexander, 1993. Deduced amino acid sequences at the haemagglutinin cleavage site of avian influenza A viruses of H5 and H7 subtypes. Arch. Virol., 30: 209-217.

Zhou, N.N., D.A. Senne, J.S. Langraf, S.L. Swenson, G. Erickson, K. Rossow, L. Lui, K.J. Yoon, S. Krauss and R.G. Webster, 1999. Rapid evolution of $H5N_1$ influenza viruses in chicken in Hong Kong. J. Virol., 73: 8851-8856.