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 9 (1): 28-31, 2010
ISSN 1682-8356

Effect of Antigen Source on the Sensitivity of Avian Influenza Virus Haemagglutination Inhibition Test

Asma Jabeen, Khalid Naeem, Zaheer Ahmed and Naila Siddique
National Reference Laboratory for Poultry Diseases, Animal Sciences Institute,
National Agricultural Research Centre, Park Road, Islamabad, Pakistan

Abstract: The effect of variation in the source of Avian Influenza Virus (AIV) antigen on the sensitivity of Haemagglutination Inhibition (HI) test was examined. For this purpose, HI antigen of AIV subtypes H5 and H7 were either prepared or procured from different sources and used to determine the antibody levels against AIV subtypes H5 and H7 in chickens, using HI test. The HI test was standardized using 30 serum samples collected from a flock experimentally vaccinated against AIV subtypes H5N9 and H7N1 under controlled conditions. The test serum samples were collected from the field among chickens vaccinated against AIV subtypes H5N9 and H7N1 vaccines and subjected to HI test using a variety of HI antigens belonging to H5 and H7 subtypes. The results reflected that the type of AIV strain used in the HI antigens affected the levels of antibody titre determination from the same samples. The effect of this variation on reliability of data regarding sero-surveillance and vaccine efficacy testing for avian influenza among poultry is discussed.

Key words: Avian influenza virus, antibody detection, haemagglutination inhibition

INTRODUCTION

Influenza virus of family Orthomyxoviridae is divided into types A, B and C on the basis of antigenic character of their nucleoprotein and matrix protein. However, only type A influenza viruses have been isolated from birds, whereas types B and C are predominantly human pathogens (Matthews, 1979). Type A influenza viruses are serologically divided into sixteen Haemagglutinin (HA) and Nine Neuraminidase (NA) subtypes (Fouchier *et al*., 2005).

Highly Pathogenic Avian Influenza Virus (HPAI) was one of the first viral diseases described in poultry (Suarez and Cherry, 2000). Influenza infections among domestic or confined birds have usually been associated with variety of disease syndrome ranging from mild upper respiratory failure to acute highly fatal generalized disease (Mo *et al*., 1997).

For Avian Influenza (AI) surveillance activities, serologic assays used to determine immune response against Avian Influenza Viruses (AIV) are Enzyme Linked Immunosorbant Assay (ELISA), Agar Gel Immunodiffusion (AGID) and Haemagglutination Inhibition (HI). Among the above, HI is the only type specific antibody detection assay. This antibody detection assay not only helps to determine field exposure of various subtypes of AIV, it is also employed to assess the level of immune response against various AI vaccines. In both the cases, it is very important to establish a reliable, reproducible and cost effective HI test procedure. Standardization of viral HA-HI tests necessarily involves all of the "arbitrary constants" that make up the test. This includes volume and concentration of red blood cells, volume of antigen, type of diluent, serum treatment, antigen-serum-cell volume ratio and incubation temperature and time. Since its inception in 1941, HI test has been subjected to variety of procedural modifications according to procedure adopted by Olsen *et al*. (2003). However due to use of stable viruses in HI test, much information has not been generated regarding the effect of antigenic variation on the sensitivity of HI test. With current situation of increased serosurveillance activities against Highly Pathogenic Avian Influenza (HPAI) all over the world, there is an acute shortage of reliable antigen and antisera for conducting HI test against different avian influenza viruses. It has, therefore, become necessary to identify antigenically stable reliable source of antigens on regular basis. While undertaking such efforts at a given local diagnostic laboratory, attempts are to be made for identifying a particular strain of AIV for antigen preparation throughout the country. The study reported here was designed to compare various HI antigens for identifying an antigenically stable type of antigen and also for standardizing a reproducible HI protocol for harmonization in avian influenza diagnosis and serosurveillance within the country.

MATERIALS AND METHODS

HI reagents: For detection of AIV subtype H5 antibodies in the serum, the antigens of AIV subtypes H5N9, H5N3,

Corresponding Author: Khalid Naeem, National Reference Laboratory for Poultry Diseases, Animal Sciences Institute, National Agricultural Research Centre, Park Road, Islamabad, Pakistan

H5N1 and H5N2 were imported from Italy along with locally prepared H5N1 antigen at National Reference Laboratory for Poultry Diseases, National Agricultural Research Council (NARC), Islamabad, Pakistan, using prevailing strain of AIV subtype H5N1 isolated from domestic Pakistani outbreaks of 2006., while for AIV subtype H7 antibodies detection all the antigens used were imported from Italy including AIV subtypes H7N3, H7N7 and H7N1.

Standardization of test antigens: For standardization of HI test, 35 serum samples were collected from a Broiler Breeder (BB) flock of 30 weeks of age, previously injected with Italian AIV H5N9 and H7N1 vaccines, twice at an interval of 3 weeks. The sampling was carried out 4 weeks post second vaccination. Out of this group, 30 AIV positive samples as determined by ELISA screening using Idexx ELISA kit (USA), were saved for future testing by HI using different subtypes of H7 and H5 antigens. These samples were used as vaccinated control.

For field evaluation of efforts to determine the variation in the source of Avian Influenza Virus (AIV) antigen on the sensitivity of Haemagglutination Inhibition (HI), 200 field serum samples received from the peripheral labs at the National Reference Laboratory for Poultry Diseases (NRLPD) of the NARC, Islamabad, Pakistan were also subjected to ELISA screening for the presence of AIV antibodies. All the BB flocks included in this study were between the age of 28- 30 weeks and previously vaccinated twice up to 25-week age with AIV subtypes of H7N1 and H5N9.

ELISA screening: All the field serum samples were first subjected to Enzyme Linked Immunosorbant Assay (ELISA) using commercially available AIV antibody detection kit (Idexx, USA), following manufacturer's instructions. The samples found positive were further tested for the detection of subtype specific HI antibody titres.

Haemagglutination inhibition test (HI): For this purpose all the control and test samples were processed for the detection of AIV antibodies using H5 and H7 antigens, following the standard protocol (Olsen *et al*., 2003). Briefly, all serum samples were heat inactivated at 55°C for 30 min. The test serum samples were two-fold serially diluted by using equal volumes (25 µl each) of 0.1 M PBS (PH 7.2) in a 96 well round bottom micro-titration plate. To these wells, 25 µl of 8HAU (Haemagglutinating Units) antigen of specific AIV subtype was added. Both negative (i.e, wells containing no antibody) and positive control (well containing known positive antibody) wells were set up in each plate in a row of the test plates. The plates were incubated for 30 min at 37°C. After incubation, 50 µl of 0.5 % chicken RBC suspension prepared in PBS were added in each well. The plates were further incubated at 37°C for 30 min. The highest dilution of each serum sample causing inhibition of haemagglutination was considered as end point. Haemagglutination Inhibition titer of each sample was expressed as reciprocal of the highest serum dilution.

RESULTS

The initial screening of 35 serum samples from a vaccinated flock (control group), revealed 30 samples as positive for AIV antibodies (Ab) when tested by ELISA (data not shown). Similarly, 126 serum samples were found positive for AIV Ab by similar ELISA out of a total of 200 test sera samples received from the field (data not shown). The 30 ELISA positive samples from AIV (H7 and H5) vaccinated control flocks were serologically evaluated using HI antigens of subtype H7 and the data are shown in Table 1. The highest Geometric Mean Titre (GMT) for HI antibody was observed against H7N7 antigen with a GMT of 445 (titre range 64-2048). With H7N3 antigen, the same test sera showed AIV antibody GMT of 194 (titre range of 32-2048), whereas by using HI antigen H7N1, the tested sera showed HI Ab GMT of 36 (titre range 8 to 256).

Serologic evaluation of AIV vaccinated control flock using HI antigens of subtype H5 is also shown in Table 1. Out of 30 samples tested, all had HI antibody showing GMT of 675 and 294, against HI antigen of H5N9 and H5N3, respectively. These titres had a range of 16-2048 and 16-1024, respectively. Upon using HI antigens of AIV subtypes H5N2 and H5N1, the same samples showed HI Ab GMT of 97 and 222, with an overall GMT range of 16-512 and 8-2048, respectively. Upon using HI antigen H5N1 (local), the test sera showed AIV Ab GMT of 84 with titre range of 16-1024.

AIV antibody distribution among 126 field samples using different AIV antigens of H7 subtype is shown in Table 2. Out of 126 field samples, 9 showed no detectable antibodies to AIV subtype and the remaining samples had antibody titre range of 4 to 2048 with a GMT of 157. Upon using H7N3 HI antigen, the same test sera showed a mean titre of 32 with titre range of 4-2048, however, in these situation 25 samples showed no detectable AIV H7 antibody titre. By using HI antigen H7N1, the tested sera showed HI antibody GMT of 29 with titre range of 4-2048, with 14 samples negative for AIV antibodies. The samples showed HI Ab GMT of 27 with titre range of 4-2048, using antigen subtype H5N9. 12 samples did not have any detectable Ab titres. When the same samples were tested using H5N3 antigen it showed HI Ab GMT of 5 with 37 samples showing no detectable Ab titres against AIV H5 and the remaining samples had AI antibody titre range of 4-128. On the other hand, upon using the HI antigen H5N2, the same samples showed AIV Ab GMT of 9. A total of 26 samples showed no detectable H5 antibody titres but the

Table 1: Serologic evaluation of samples from AI vaccinated control flock using HI antigens of subtype H7 and H5

Antigen type	No. of samples tested	No of positive samples under each titre group											HI* GMT**
		2	4	8	16	32	64	128	256	512	1024	2048	
H7N7 (Italian)	30	-	-	-	-	-	3	4	5	6	8	4	445
H7N3 (Italian)	30	-	-	-	-	1	9	8	3	3	3	3	194
H7N1 (Italian)	30	-	-	1	6	12	9	1	1	-	-	-	36
H5N9 (Italian)	30	-	-	-	1	-	-	2	6	4	5	12	675
H5N3 (Italian)	30	-	-	-	1	-	4	3	9	6	7	-	294
H5N2 (Italian)	30	-	-	-	1	4	10	8	6	1	-	-	97
H5N1 (Italian)	30	-	-	1	-	4	2	5	8	2	6	2	222
H5N1 (Local)	30	-	-	-	4	4	7	9	4	1	1	-	84

*Haemagglutination Inhibition (HI), **Geometric Mean Titre (GMT)

Table 2: Serologic evaluation of field samples using HI antigens of AIV subtypes H7 and H5

Antigen type	No. of samples tested	No of positive samples under each titre group											HI* GMT**
		2	4	8	16	32	64	128	256	512	1024	2048	
H7N7 (Italian)	126	9	8	9	6	6	10	5	15	13	7	38	157
H7N3 (Italian)	126	25	17	15	5	3	8	13	11	12	4	13	32
H7N1 (Italian)	126	14	19	16	13	13	8	17	9	3	6	8	29
H5N9 (Italian)	126	12	19	15	7	27	12	10	11	7	3	3	27
H5N3 (Italian)	126	37	26	30	14	9	5	5	-	-	-	-	5
H5N2 (Italian)	126	26	20	28	21	8	7	8	7	-	1	-	9
H5N1 (Italian)	126	23	24	25	9	8	15	8	3	6	2	3	13
H5N1 (Local)	126	30	38	34	7	8	3	1	2	2	1	-	5

*Haemagglutination Inhibition (HI), **Geometric Mean Titre (GMT)

remaining samples showed HI Ab titre range from 4-1024. Using HI antigen H5N1, the samples showed HI Ab GMT of 13 with 23 samples showing no Ab titres, however, a titre range of 4-2048, was observed in the positive samples. Similarly when HI antigen H5N1 (local isolate) was used to test these samples, GMT of AIV Ab declined to 5. In this case, 30 samples showed no detectable antibody titres and rest of the samples had a titre range of 4-1024.

DISCUSSION

The Haemagglutination (HA) and Haemagglutination Inhibition (HI) tests have been subjected to different procedural modification over the past many years. There are two major considerations in establishing standardized HA-HI procedures. First, a study of diagnostic viral HA-HI tests needs to be performed in an effort to render them more sensitive and reliable. Secondly, a standardized protocol is needed to serve as a reference when comparing results in one laboratory with those in another (Hierholzer and Suggs, 1969).

In the current scenario of avian influenza diagnosis and surveillance, it has become important to improve the sensitivity and reliability of HI test especially for comparing results of peripheral AI surveillance labs with the central reference labs within a country or between national and international reference labs. This information can help in evaluating the epidemiology of AI, leading to decisions regarding culling of affected or carrier flocks. In addition to the above, sensitivity of test becomes further important in case of evaluation of post-vaccination immune response to determine the effectiveness of any particular AIV vaccines.

In the present study, it was revealed that there is a variation in the detection of antibody titres upon using different source of AIV HI antigen, both in case of testing antibody response against AIV subtypes H7 and H5 vaccines. The data presented for H7 antibody detection indicates drastic variation among three sets of antigen used for testing the same serum samples. It appears as if this variation is based on the variable N-type used, however, it may not be true due to the fact that the vaccination used in the tested flocks was against H7 of subtype H7N1, whereas the highest antibody titres seen in the present study were detectable by using H7N7 antigen.

It was also seen that similar variability of antibody detection existed among the control flocks given H7 vaccine. The overall antibody levels are relatively higher in the control group, which may be on account of better vaccination practices or because of better health management of the experimentally raised flock. On the other hand data presented for AIV subtype H5 indicates drastic variation among five sets of antigen used for testing the same serum samples. Here the highest titres among H5 antigens were detected using H5N9 antigen. This may be due to the fact that the flock was vaccinated with H5N9 strain of AIV for the determination of post- vaccination response. The overall GMT was significantly lower than the control group. The antibody response detected by using H5N9 Ag showed the highest response of GMT 675, this again reflects that as

the flocks were given vaccine against H5N9 strain, it may have some effect on the detection of antibody response using Ag of same subtype. This phenomenon, however, does not appear to be working in case of H7 vaccine testing, as the flocks were given H7N1 vaccine but antibody titres were lowest when tested with the same antigen.

It has also been reported that intact AI virus as compared to isolated HA units would give erroneous results when used in seroepidemiological studies (Lu *et al*., 1982), however, it may not be applicable in our situation where the variation in titre determination is based on different N types of a particular subtype of AIV and all types of antigens originated as a whole virus antigen. The variation in Ab titre determination may also be due to some differences in the process of antigen preparation, particularly the method of inactivation. Therefore, it could be anticipated that the method of inactivation of locally prepared antigen may be different from imported antigen, giving variable results for determination of antibody titres in this situation as well. The overall evaluation of the data in this study reflects that variation in antigen source or subtype does affect the detection of immune response against vaccine. The same may be true while carrying out sero-surveillance to determine exposure levels in healthy flocks. Therefore, if a specific subtype of HI antigen is not identified for AI-diagnostic work, it will be difficult to avoid variability in results from different labs across the country. This can obviously affect the decisions to be undertaken in case of serosurveillance results and immunization evaluation reports against LPAI and HPAI of H5 or H7 subtypes, respectively. It is, therefore, advisable to use a single source of HI antigen for AIVs at least within the serosurveillance network of a country for creating harmony in testing procedures leading to effective decision making.

REFERENCES

Fouchier, R.A., V. Munster, G.F. Rimmelzwaan, B. Olsen and A.D. Osterhaus, 2005. Characterization of a novel influenza A virus haemagglutinin subtype (H16) obtained from black-headed gulls. J. Virol., 79: 2814-2822.

Hierholzer, J.C. and M.T. Suggs, 1969. Standardized viral haemagglutination and haemagglutination-inhibition tests. Appl. Microbiol., 18: 816-833.

Lu, B., R.G. Webster and V.S. Hinshaw, 1982. Failure to Detect Haemagglutination-Inhibiting Antibodies with Intact Avian Influenza Virions. Infect. Immun., 38: 530-535.

Mo, I.P., M. Brugh, O.J. Fletcher, G.N. Rowland and D. Swayne, 1997. Comparative pathology of chickens experimentally inoculated with avian influenza viruses of low and high pathogenicity. Avian. Dis., 41: 125-136.

Matthews, R.E.F., 1979. Classification and nomenclature of viruses. Third report of international Committee on taxonomy of viruses.

Olsen, C.W., A. Karasin and G. Erickson, 2003. Characterization of swine like Reassortant H1N2 influenza virus isolated from wild duck in United States. Virus Res., 93: 115-121.

Suarez, D.L. and S. Cherry, 2000. Immunology of avian influenza virus. J. Develop. Comp. Immunol., 24: 269-283.