Antigenic characterization of avian influenza H9 subtype isolated from desi and zoo birds

Farrukh Saleem,^{1,2} Muhammad Mahmood Mukhtar,² Khushi Muhammad,² Muhammad Javed Arshed¹ and Muhammad Abubakar¹

¹National Veterinary Laboratory, Park Road, Islamabad; ²Department of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan

Abstract

Avian influenza is a viral infection which affects mainly the respiratory system of birds. The H9N2 considered as low pathogenic avian influenza (LPAI) virus and continuously circulating in poultry flocks causing enormous economic losses to poultry industry of Pakistan. As these viruses have RNA genome and their RNA polymerase enzyme lacks proof reading activity which resulted in spontaneous mutation in surface glycoproteins (HA and NA) and reassortment of their genomic segments results in escape from host immune response produced by the vaccine. Efforts made for the isolation and identification of avian influenza virus from live desi and zoo birds of Lahore and performed antigenic characterization. The local vaccines although gives a little bit less titer when we raise the antisera against these vaccines but their antisera have more interaction with the local H9 subtype antigen so it gives better protective immune response. Infected chicken antisera are more reactive as compare to rabbit antisera. This shows that our isolates have highest similarity with the currently circulating viruses. These results guided us to devise a new control strategy against avian influenza viral infections. The antigenic characterization of these avian influenza isolates helped us to see the antigenic differences between the isolates of this study and H9 subtype avian influenza viruses used in vaccines. Therefore, this study clearly suggests that a new local H9 subtype avian influenza virus should be used as vaccinal candidate every year for the effective control of influenza viral infections of poultry.

Introduction

Influenza viruses are negative sense segmented genome RNA virus belongs to family orthomyxoviridae. Influenza viruses are divided into 3 types i.e., A, B and C. Only type A

viruses are present in hosts other than humans. Influenza A viruses composed of 8 gene segments (PB2, PB1, PA, HA, NP, NA, M and NS) encoding 11 viral proteins. Type A influenza viruses are further subdivided into subtypes on the basis of their surface protein Hemagglutinin (HA) and Neuraminidase (NA). Among humans only three subtypes (H1N1, H1N2, H3N2 and H5N1) are currently circulating.¹

All sixteen HA and nine NA are present in wild aquatic birds. Out of sixteen different HA type only two (H5 and H7) are included in highly pathogenic and others are low pathogenic. The influenza viruses causing disease in avian species are known as avian influenza virus. It was first recognized in the early 1900s in Italy.^{2,3} The H9N2 considered as low pathogenic avian influenza (LPAI) virus and continuously circulating in poultry flocks causing enormous economic losses to poultry industry of Pakistan.⁴ Diagnosis of AI is preferably made by virus isolation *via* inoculation of embryonated chicken eggs (ECE), demonstration of haemagglutinating activity and characterization of avian influenza virus (AIV) by agar gel immunodiffusion test.⁵ Serological detection of infection in the host is important for early diagnostic detection program for developing appropriate control strategies.⁶

As these viruses have RNA genome with RNA polymerase enzyme lack proof reading activity which resulted in spontaneous mutation in surface glycoproteins (HA and NA) and reassortment of their genomic segments results in escape from host immune response produced by the vaccine.⁷ The vaccine does not give protection against specific virus after some time because the variation come among the virus strain using for vaccination and the virus prevailing in the field. This is the reason that every year we require a new candidate virus for vaccine preparation. Nguyen *et al.*⁸ performed antigenic characterization in this scenario to develop the control strategy.

Materials and Methods

Sample collection

A total of 480 cloacal swabs and dead bird tissue samples for the isolation of avian influenza virus were collected from desi-bird retail markets like Lohari, Lakshmi, Bhati, Samanabad and Mughalpura area of Lahore, the wild bird market (Tolent Market) and Lahore Zoological Garden (Lahore Zoo). The material used for the sampling was sterilized cotton swabs, scissor, glycerol medium, sterile plastic screw capped tubes, ceiling bags, permanent marker, and the ice box with ice bags for the proper transportation of the samples.

Correspondence: Muhammad Abubakar, National Veterinary Laboratory, Park Road, Islamabad, Pakistan.

E-mail: hayee41@gmail.com

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Samples were properly labeled and transported in glycerol medium to the laboratory by placing them in ice box. The specimens were processed within 24 hr or stored at 4°C for 2-4 days. The samples were processed by placing them on the vertex machine and after mixing of these samples the samples were centrifuged on 4000 rpm for 5 min. After centrifugation the supernatants were collected and inoculated in the embryonated eggs.

Cultivation of virus

A total of 180, nine-day-old chicken embryonated eggs were obtained from Big Bird Hatchery, Lahore. Chorio-allantoic sac (CAS) route was used for inoculation of samples. The eggshell was disinfected with 70% methyl alcohol and marked area was painted with tincture iodine. A small hole was made and 0.2 mL quantity of fluid was injected. The drilled area of each egg was sealed with sterilized molten wax and the eggs were re-incubated with control eggs at 37°C with relative humidity of 60-70%. The inoculated eggs and control eggs were candled 24 h post-inoculation and the dying embryos within 24 h of inoculation were discarded and rest of the eggs are further incubated for the next 24 h. During candling those eggs were showing the dead in shell state discarded and the eggs showing the clear live embryo with moving head in the shell were separated and kept for further incubation. Those dying after 24 h post inoculation and all survivor after 60 h post inoculation were chilled at + 4°C for 8 h in refrigerator. After 48 h following the inoculation the embryonated eggs were harvested and fluid was centrifuged and checked for the hemagglutination.

Spot agglutination test

The harvested amniotic fluid was tested for its hemagglutinating activity by spot agglutination test. This test is the preliminary test for

the identification of just hemagglutinating viruses. Equal quantity of 5% RBCs and harvested amniotic fluid was mixed on a clean glass slide and allowed to react with each other for 3 min. The agglutination of RBCs within 3 min showed the positive agglutinating virus and then that fluid was stored for further confirmation of avian *influenza* H₉ subtype virus.

Hemagglutination test (HA)

Test protocol

Fifty μ L of diluents (PBS) were added in each of the 12 wells of micro titer plate in a series. Fifty μ L of amniotic fluid was added with the help of microtiter pipette and mixed well giving 2 fold dilution (1:2) in the first well of each series up to 11th well. Fifty μ L of the suspension was discarded from the 11th well. Fifty μ L of the 1% RBCs from different sources were added to each well from 1-12. In each series 12th well acted as erythrocyte suspension control as no antigen was added. The plates were incubated at 25°C and checked 12th well as control for complete setting of RBCs.

Virus neutralization test (VNT)

The virus neutralization test was performed to confirm the viral isolates. Virus and serum were mixed under appropriate condition and then inoculated into embryonated eggs. The presence of un-neutralized virus may be detected by reactions such as hemagglutination. The loss of infectivity was bought about by interference by the bound Ab with any one of the steps leading to the release of the viral genome into the host cells.

Avian *influenza* virus antigen rapid test (VART)

This test performed using the specialized kit (AIV Antigen Rapid Test Kit, Manufactured by ANIGEN, Animal Genetics, Inc. H5 AIV Antigen Rapid Test Kit, manufactured by ANIGEN, Animal Genetics, Inc). By using above mentioned kit, the isolates confirmed by differentiating between the Newcastle Disease Virus and the avian *influenza* virus and by using another kit differentiation done between the H5 and H9 Avian *Influenza* Virus.

Hemagglutination inhibition (HI)

Test protocol (WHO, Manual of Avian *Influenza*, WHO/CDS/CSR/NCS/2002.5 Rev.1)

Twenty-five μ L of diluent was added in a 96 well micro-titration plate in each of the 12 wells in a row. Twenty-five μ L serum was added and thoroughly mixed in the 1st well of each series. Twenty-five μ L of the diluted serum was transferred from the 1st well to the 2nd well and made the 2 fold dilution. Till the

10th well this process was repeated from which 25 μ L of the diluted serum was discarded. Thus no serum was added to 11th and 12th well. From the first to 11th well, 25 μ L of antigen containing 1, 4 and 8 HA units were added and mixed well. The 11th well contained no serum and acted as antigen control. The plates were incubated at 37°C to allow the completion of antigen antibody reaction. Thirty min incubation time was given to plates. Fifty μ L of 1% washed chicken RBCs were added to each well and the plates were further incubated for 30 min. There would be complete hemagglutination in 11th well (antigen control) and no hemagglutination in 12th well (diluent control).

Results

Total 480 cloacal and tracheal samples collected from the bird retail markets, Tolenten market, Lahore Zoo and from the broiler chicken. Out of these 480 samples, 136 cloacal samples collected from the desi bird retail markets, 170 cloacal samples collected from different wild birds from the Tolenten market, 134 cloacal samples collected from Lahore Zoo and then 40 tracheal samples collected from broiler chicken (Table 1).

As the samples were processed and inoculated them in 9 days old embryonated eggs for the cultivation of the virus, after harvesting the fluid total 4 positive (repetition) samples were positive out of 480 samples which were about 0.83%. The fluid giving the hemagglutination was further tested for the titer by performing the HA test. All the positive samples properly marked and stored at 4°C for further processing. These four positive samples were founded from different samples collected from different areas of Lahore. The one positive sample out of 136 samples which is 0.86% collected from the desi bird retail markets, one positive from the

turkey out of 170 samples which is 0.58% were collected from the tolenten market, the 3rd positive sample from the peafowl out of 134 samples which is 0.74% collected from the Lahore Zoo and the 4th positive sample form the broiler farm out of 40 samples which is 2.60% (Table 1).

The virus neutralization test (VNT) was performed to confirm the viral isolates. Virus and serum were mixed under appropriate condition and then inoculated into embryonated eggs. The presence of un-neutralized virus was detected by hemagglutination activity of the harvested AAF. The loss of infectivity was bought about by interference by the bound Antibodies with *influenza* virion.

The AIV Antigen Rapid Test Kit and the H5 AIV Antigen Rapid Test Kit used to test all the four positive amnio-allantoic fluid (AAF) harvested from the embryonated eggs. By using these test kits the four positive isolates were differentiated from the Newcastle Disease virus and H5 subtype avian *influenza* virus.

The fluids giving very low titers were re-inoculated in the embryonated eggs and given 1st passage for the cultivation of the virus. After 2nd passage the virus titer was again checked by the hemagglutination test. The titer of the 2nd passage was 5, 6, 5 and 6 for desi chicken, turkey, peafowl and chicken respectively (Table 2). The best titer was from the tracheal samples taken from chicken as compare to the cloacal samples (Figure 1). The virus titer in this passage was enough for further processing so then we performed Hemagglutination Inhibition test with known antisera, which we have raised by giving the 1st short to the rabbits on the 0 day and then 2nd short on 21st day, on the same day the serum was collected to check titer of antibodies. Then the antisera were collected by bleeding the rabbits 21 days after boosting (42nd day).

A total of 6 serum samples were obtained

Table 1. Isolation of the h9 subtype avian influenza viruses.

No. of Samples	Location	Specie	+ ve Samples	% +ve
136	Desi Bird RetailMarket	Chicken	1	0.86
170	Tolenten Market	Turkey	1	0.58
134	Lahore Zoo	Pea fowl	1	0.74
40	Poultry Farm	Chicken	1	2.60
Total 480			4	0.83

Table 2. Hemagglutination titer of the harvested fluid.

Viruses	HA Titer
A/Desi Chicken/Pakistan/Micro-1/2009	5
A/Turkey/Pakistan/Micro-2/2009	6
A/Pea Fowl/Pakistan/Micro-3/2009	5
A/Chicken/Pakistan/Micro-4/2009	8

from 6 rabbits vaccinated against killed or inactivated H9 avian *influenza* vaccine. Two rabbits out of 6 rabbits were vaccinated with one imported and two local vaccines. By inoculating the known commercially available inactivated or killed H9 subtype avian *influenza* vaccines, the antisera was raised and used for further tests.

When the antisera collected for further tests 1stly checked the antibody titer in the serum against the H9 subtype avian *influenza* virus. The titer of the 1st antisera raised against the imported vaccine (Merial Group) was 256, 2nd antisera raised against the local vaccine (Bio-Avian Vaccine) that was 128 and then the antibody titer of the 3rd antisera raised against the local vaccine (Ottoman vaccine) was 128. The H9 subtype avian *influenza* virus infected chicken antisera was having 256 titer.

After confirming the H9 isolates these isolates preceded further for the antigenic characterization which has been performed through Hemagglutination Inhibition test by interacting the virus with 6-Antisera raised in the rabbits and also with the reference chicken antisera. In the antigenic characterization we have seen when the first isolate A/Desi Chicken/Pakistan/Micro-1/2009 was interacted with the serum raised in the rabbits against the imported vaccine the titer was 32 and with the local two vaccines were 64 and 32 where as the highest titer seen when the same isolate interacted with the chicken antiserum which was 256. Similarly the 2nd isolate A/Turkey/Pakistan/Micro-2/2009 when interacted with the serum raised in the rabbits against the imported vaccine the titer was 128 and with the local two vaccines were 512 and 512 where as the highest titer seen when the same isolate interacted with the chicken antiserum which was 1024. The 3rd isolate A/Pea Fowl/Pakistan/Micro-3/2009 was interacted with the serum raised in the rabbits against the imported vaccine the titer was 64 and with the local two vaccines were 128 and 32 where as the highest titer seen when the same isolate interacted with the chicken antiserum which was 256 and when the last isolate A/Chicken/Pakistan/Micro-4/2009 was when interacted with the serum raised in the rabbits against the imported vaccine the titer was 64 and with the local two vaccines were 256 and 256 where as the highest titer seen when the same isolate interacted with the chicken antiserum which was 1024 (Table 3).

Discussion

Type A *influenza* viruses are further subdivided into subtypes on the basis of their surface protein (Hemagglutinin HA and Neuraminidase NA). There are sixteen differ-

ent HA (H1-H16) and nine different NA (N1-N9) surface glycoproteins are known till now. *Influenza* A viruses composed of 8 gene segments (PB2, PB1, PA, HA, NP, NA, M and NS) encoding 11 viral proteins.⁹ Diagnosis of AI is preferably made by virus isolation via inoculation of embryonated chicken eggs (ECE), demonstration of hemagglutinating activity and characterization of AIV by hemagglutination inhibition test.

Serological detection of infection in the host is important for early diagnostic detection program for developing appropriate control strategies⁶. These viruses have RNA genome with RNA polymerase enzyme lack proof reading activity which resulted in spontaneous mutation in surface glycoproteins (HA and NA) and reassortment of their genomic segments results in escape from host immune response produced by the vaccine⁷. This is the reason that every year we require a new candidate virus for vaccine preparation. Therefore, antigenic characterization of field isolates from live desi and zoo birds is very important because most of research work already performed based on the isolations of avian *influenza* virus from commercial poultry.

About 90 species from some 12 of the 50 Orders of birds have yielded influenza viruses, the number, variety and widespread distribution of *influenza* viruses has been far greater in waterfowl, Order Anseriformes, than in other birds.¹⁰ In the surveys listed by Stallknecht,¹⁰ a total of 21,318 samples from all species resulted in the isolation of 2317 (10.9%) viruses. Of these samples 14,303 were

from birds of the Order Anseriformes and yielded 2173 (15.2%) isolates. The next highest isolation rates were 2.9 and 2.2% from the Passeriformes and Charadriiformes, respectively and the overall isolation rate from all birds other than ducks and geese was 2.1%.¹¹

In this study the selected area or the place of sampling is mainly from Lahore and the samples for the isolation of H9 subtype avian *influenza* focused from the live desi bird markets of Lahore and the zoo birds from Lahore Zoo. The selection of this area for study has been done because no or very limited work has been done on the healthy desi and wild birds.

There were total 4 isolates obtained out of total 480 samples. These isolates were A/Desi Chicken/Pakistan/Micro-1/2009 isolated from the desi chicken, one out of 136 samples which is only 0.86%. Similarly the 2nd isolate A/Turkey/Pakistan/Micro-2/2009 isolated from the turkey a wild bird, out of 170 samples only 1 sample was positive which was only 0.58%. The third isolate A/Pea Fowl/Pakistan/Micro-3/2009 isolated from the pea fowl, out of 134 samples only 1 sample was positive which was only 0.74% and the fourth isolate A/Chicken/Pakistan/Micro-4/2009 was isolated from chicken, out of 40 samples only 1 sample was positive which was 2.60% of total. The rate of virus incidence is very much low depending on different physical and environmental situations. Like in summer and winter there are more chances of and more burden of virus infection as compare to the virus isolation rate in other seasons.^{12,13}

Due to lack of proof reading activity results

Figure 1. Hem-agglutination inhibition titer showing the antigenic interrelation of H9 subtype virus isolated in lahore in 2009.

Table 3. Hemagglutination inhibition activity of pakistani h9 subtype avian influenza viruses.

HAI reactivity of reference Viruses	Rabbit antiserum against H9 vaccines			
	Merial	Bio-Avian	Ottoman	Chicken Antiserum
A/Desi Chicken/Pakistan/Micro-1/2009	32	64	32	256
A/Turkey/Pakistan/Micro-2/2009	128	512	512	1024
A/Pea Fowl/Pakistan/Micro-3/2009	64	128	32	256
A/Chicken/Pakistan/Micro-4/2009	64	256	256	1024

in spontaneous mutation and reassortment of their genomic segments, every year we need a new candidate virus for vaccine preparation. For this reason antigenic characterization of field isolates from live desi and zoo birds is also important.

Most of research work already performed based on the isolations of avian *influenza* virus from commercial poultry. Therefore, we are focusing on the desi and zoo birds to see the presence of *influenza* virus infection in these birds. The main objectives of this study is the isolation and Identification of H9 subtype avian *influenza* viruses from live desi and zoo birds also to perform the antigenic characterization of these avian *influenza* virus isolates.

In the antigenic characterization we have seen when the first isolate A/Desi Chicken/Pakistan/Micro-1/2009 was interacted with the serum raised in the rabbits against the imported vaccine the titer was 32 and with the local two vaccines were 64 and 32 where as the highest titer seen when the same isolate interacted with the chicken antiserum which was 256. Similarly the 2nd isolate A/Turkey/Pakistan/Micro-2/2009 when interacted with the serum raised in the rabbits against the imported vaccine the titer was 128 and with the local two vaccines were 512 and 512 where as the highest titer seen when the same isolate interacted with the chicken antiserum which was 1024. The 3rd isolate A/Pea Fowl/Pakistan/Micro-3/2009 was interacted with the serum raised in the rabbits against the imported vaccine the titer was 64 and with the local two vaccines were 128 and 32 where as the highest titer seen when the same isolate interacted with the chicken antiserum which was 256 and when the last isolate A/Chicken/Pakistan/Micro-4/2009 was when interacted with the serum raised in the rabbits against the imported vaccine the titer was 64 and with the local two vaccines were 256 and 256 where as the highest titer seen when the same isolate interacted with the chicken antiserum which was 1024. The results of the hemagglutination inhibition test are shown in the Table 2. In the interaction of 1st isolated virus it is observed that the 1st titer which is for antisera raised against imported vaccine giving the minimum titer as compare to the two antisera raised against other local vaccines and if we see the H9 infected chicken antisera giving the highest titers. By studying these results we can say that the locally isolated virus is more antigenically similar that is why giving more titer against the antisera which were raised against local vaccines but giving less titer against the antisera which were raised against imported vaccine. Here another thing which is more noticeable is that the antisera raised in the rabbits giving less titer as compare to H9

infected chicken antisera.

It is important that the low titer of these isolates may be due to the antigenic drift present in this virus. Almost similar results have been shown by the Guan *et al.*¹² which discuss that there was antigenic drift that is present in the *influenza* virus that is why the antisera raised against the vaccinal strain may unable to give protective titer.

Conclusions

As these viruses have RNA genome and their RNA polymerase enzyme lacks proof reading activity which resulted in spontaneous mutation in surface glycoproteins (HA and NA) and reassortment of their genomic segments results in escape from host immune response produced by the vaccine.

This is the reason that every year we require a new candidate virus for vaccine preparation. We have made an effort to isolate and identify avian *influenza* viruses from live desi and zoo birds of Lahore and performed antigenic characterization. In this way, we have been able to know the exact status of avian *influenza* virus strains present in the desi and zoo birds. We also have seen that the imported vaccine have less interaction with the local strains and gives less protective titer although it gives best titers when we raise antisera against imported vaccine. The local vaccines although gives a little bit less titer when we raise the antisera against these vaccines but their antisera have more interaction with the local H9 subtype antigen so it gives better protective immune response. By this study we have seen that antisera obtained from infected chicken give more antibody titer as compare to antibody raised in the rabbits. Infected chicken antisera are more reactive as compare to rabbit antisera. This shows that our isolates have highest similarity with the currently circulating viruses.

All above results helped us to devise a new control strategy against avian *influenza* viral infections present in these local birds. The antigenic characterization of these avian *influenza* isolates helped us to see the antigenic differences between the isolates of this study and H9 subtype avian *influenza* viruses used in vaccines. Therefore, this study clearly suggests that a new local H9 subtype avian *influenza* virus should be used as vaccinal candidate every year for the effective control of *influenza* viral infections of poultry.

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