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Viral Shedding Evaluation is Critical for Determining Efficacy of Avian Influenza (H9) Vaccines in Broiler Chickens

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ABSTRACT

Viral shedding evaluation is critical for determining the efficacy of avian influenza (H9) vaccines in broiler chickens. This study was undertaken to evaluate the comparative efficacy of commercial and self-prepared AIV H9 vaccines for their potential to stop or reduce viral shedding post challenge infection (PCI) and to develop humoral immunity. A total of 5 groups A, B, C, D and E comprising of 20 broiler birds each were made. Groups A, B and C were vaccinated on 6th day of age with imported (Ivac), local (Lvac) and self-prepared (Spvac) vaccines, respectively. Group D (virus challenged) and E (non- vaccinated/ non-challenged) were kept as control groups. Virus shedding in cloacal swabs, taken on 15 consecutive days PCI from all the groups (25th day, 100 $EID_{50,}$ intranasal) were tested through Real-Time RT-PCR. Humoral immune response were also measured by haemagglutination inhibition (HI) test using blood samples collected at an interval of 5 days till the age of 40 days. In this study, none of the vaccines found effective to prevent virus shedding however statistically, the difference in viral load was non-significant ($P<0.5$) within the local vaccinated groups while significant ($P>0.5$) when compared local with imported vaccine and control groups. Post challenge infection, virus shedding started on 3rd day in group D while in all vaccinated groups it was (A: 730, B: 470 and D: 430 copies) on 4th day PCI and was maximum on 6 days PCI (A: 1940, B:1645 and D: 1550 copies) followed by decline. The shedding of virus stopped on 12th day PCI in groups (B and C) while it stopped on 15th day PCI in group A, however in group D, it continued till the end of trial. In present study, antibody titers (geometric mean titer-GMT) showed increasing trends (48.5 to 55.5) in all vaccinated groups ($p>0.05$) until challenge infection followed by decrease in antibody titer (22.6 to 27.9) for 5 days PCI. This study indicated, all the vaccines found effective in reducing virus shedding and induction of humoral immunity however local vaccines presented robust reduction compared to imported vaccine and can be used preferably due to cost effectiveness and availability to combat avian influenza H9 in poultry birds.

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Avian influenza H_9 virus vaccine, Efficacy, Virus shedding quantitation, Real time RT PCR, Humoral immunity

INTRODUCTION

Poultry industry is considered as one of the most vibrant segments of agriculture in Pakistan and is the source of employment for more than 1.5 million people (Khalid, 2020). This industry has been suffering from many bacterial or viral pathogens that results in significant economic losses. Among viral diseases, avian influenza virus (AIV) is a contagious disease of poultry. Avian influenza virus is a negative sense RNA virus having segmented genome within Orthomyxoviridae family (Jordan *et al*., 2018). Influenza A viruses are classified on the basis of surface glycoprotein haemagglutinin (18HA) and neuraminidase (11 NA) into various subtypes (Iqbal *et al*., 2013). On the basis of pathogenicity, AIV are categorized as high (HPAIV) and low path (LPAIV). The high path AIV (H5 andH7) are nor reported since 2008 in Pakistan however LPAIV (H9) is endemic (Cui *et al*., 2017; Ebrahim and Seioudy, 2020).

Initially, only HPAIV (H5 and H7) grabbed the attention of the researchers and industry but now economic impact of LPAIV (H9) in many countries of world including Pakistan has been established. In Pakistan, H9 subtype of AIV is causing economic losses in terms of poor growth rate, low production performances, high medication cost, increased morbidity and superinfection by bacteria

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(Shaukat *et al*., 2016; Sultan *et al*., 2017). Avian influenza is responsible for 2.2 billion rupees annual loss in poultry sector (Khan *et al*., 2021). The role of several viral and bacterial pathogens to increase severity of AIV H9 infection in poultry flocks has been reported and these cause high mortality (up to 65%) and drop in egg production (up to 50%) (Hasni *et al*., 2021). To control AIV H9 infection, many countries have adopted vaccination strategies to minimize the economic losses. Vaccination strategies are focused to induce protective immunity against the field exposure of viruses and to reduce the virus spreading to susceptible flocks.

In Pakistan, H9 subtype of avian influenza virus is endemic since 1998 (Ahad *et al*., 2013). Poultry producers are vaccinating their flocks by using both local and imported vaccines. In spite of spending lot of money on vaccination, problem of AIV H9 infection is still present all over Pakistan which may due to high tendency of mutation in influenza viruses (vaccine mismatch) (Swayne *et al*., 2011). Local vaccine producers and importers always claim about the effectiveness of their vaccines on the basis of humoral immunity and challenge protection data without addressing virus shedding (Ebrahim and Seioudy, 2020). Virus shedding evaluation is crucial tool for the evaluation of vaccine efficacy. Real time RT PCR has been increasingly used for the detection of viruses based on its sensitivity, specificity and quantification of infectious particle (Tavakkoli *et al*., 2011).

Keeping in view the economic impact of AIV H9 infection, this study was undertaken to evaluate the comparative efficacy of commercial and self-prepared AIV H9 vaccines for their potential to stop or reduce viral shedding post challenge infection (PCI) and to develop humoral immunity in broiler chickens.

MATERIALS AND METHODS

In this study, two commercial avian influenza (H9) vaccines (imported: Ivac and local: Lvac) and one self-prepared vaccine (Spvac) containing AIV H9 field 2019 isolate (MN994294) was evaluated for their potential to stop or reduce viral shedding post challenge infection (PCI) and to develop humoral immunity in broiler chickens.

Experimental groups

A total of 5 groups A, B, C, D and E comprising of 20 broiler birds each were made. Groups A, B and C were vaccinated on 6th day of age with imported (Ivac), local (Lvac) and self-prepared (Spvac) vaccines, respectively as per Pakistan Poultry Association recommended vaccination schedule. Group D (only challenged, non-vaccinated) and E (non-vaccinated/ non-challenged) were kept as control groups.

Quantitation of virus shedding by real-time RT-PCR

Virus shedding in cloacal swabs, taken on 15 consecutive days from all the groups PCI (25th day, 100 $EID_{50,}$ intranasal) were tested through Real-Time RT-PCR (Tavakkoli *et al*., 2011). Total RNAs were extracted from cloacal swabs using QiaAmp® kit (Cat#51306) as per manufacturer's instructions and tested. Viral load in cloacal swabs were quantified through real time RT-PCR using previously used specific primers (H gene) and Taq Man probes labelled with 6-Carboxyflouriscen (FAM) reporter dye at 5' end and quencher (6-carboxytetramethylrhodamine) at 3' end (Shabat *et al*., 2010) (Table I). Extracted RNA was subjected for amplification using one step real time RT- PCR kit (Agpath-ID™) following manufacturer's instructions. Reaction mixture was prepared in 25 µL final volume (2 µL RNA and 23 µL master mix containing 12.5 ul of RT-PCR buffer, 1 ul of RT PCR Enzyme mix, 1 ul of each (forward and reverse) primer, 1 ul of probe and 6.5 ul of nuclease free water.). Thermal profile of H9 subtype was as: 45ºC for 10 min, 95 ºC for 10 min followed by cycling steps of 95 ºC for 15 sec, 60 ºC for 45 sec repeated for 40 cycles. The assay was performed on Bio Rad real time PCR system (Tavakkoli *et al*., 2011). HA gene copy number was quantified by standard curve and was used to present the quantitative (qPCR) data in genome copy number. Standard curve was obtained by serial tenfold dilution of standard sample after quantification of RNA by nano drop spectrophotometer. Standard curve was plotted using Microsoft excel.

Table I. Real time primers and probe sequence for H9.

Primer/ Probe	Sequence (5'-3')
H9F	GGAAGAATTAATTATTATTGGTCGGTAC
H9R	GCCACCTTTTTCAGTCTGACATT
H9Probe	FAM-5_-AACCAGGCCAGACATTGCGAGTAA-GATCC-3_-TAMRA

Processing of blood samples to study humoral immunity

Humoral immune response was also measured by haemagglution inhibition (HI) test (OIE, Terrestrial Manual, 2009) using blood samples collected from all the experimental groups at an interval of 5 days (5th, 10th, 15th, 20th, 25th, 30th, 35th) till the age of 40 days. HI titers were determined and geometric mean titer (GMT) were calculated (Ali *et al*., 2016).

Statistical analysis

Collected data was analyzed statistically using SPSS software by repeated measures and one way ANOA.

RESULTS

Virus shedding and viral load quantification

No clinical signs were noticed in all the vaccinated groups except in challenged control group D where clinical signs started on 3rd day PCI and persisted for 7 days however no mortality was recorded in either vaccinated or control groups. To determine the potential of various vaccines for preventing or reducing virus shedding PCI with respect to time, Real time RT-PCR (qRT-PCR) was performed. In current study, none of the vaccine stopped the virus shedding. Virus shedding started on 4th day PCI except control group where shedding was evidenced on 3rd day PCI. Among vaccinated groups, highest viral load, mean virus copies per µg, was observed in A (Ivac; 730 copies) group followed by B (Lvac: 470 copies) and C (Spvac: 430 copies). The viral load was around 10 time more in control group D (4854 copies). Viral load continued to increase and was maximum on 6th day PCI. Highest viral load was observed in A: 1940 copies followed by B: 1645 copies whereas the viral load was 100 times more in non-vaccinated D group (28152224 copies). On day 7, virus load was declined in all vaccinated groups, A: 1600 copies, B: 1278 copies). Continuous declining trend in viral load was observed on 8th day PCI (A: 1023, B: 620, C: 700). Similar declining trend in virus shedding was noted in all the experimental groups (Table II). Complete cession of virus shedding observed on 12th day PCI in local (Lvac and Spvac) B and C vaccinated group however, in group A, virus shedding stopped on 15th day PCI. Whereas in virus challenged group (Group D) virus shedding was not stopped till the end of trial (15th day PCI). Birds of group E (NC) remained negative throughout the trial and did not shed virus. Statistically there was non-significant differences ($p<0.05$) among groups vaccinated with local vaccines while there was a significant difference ($p>0.05$) observed when compared local vaccines (Lvac and Spvac) with imported (Ivac) vaccine. There was also significant ($p>0.05$) difference observed in control groups (D and E) when compared with all the vaccine groups (Table II).

Comparison of humoral immune response in experimental groups

In experimental birds, anti-AIV H9-HI antibody titers were measured to compare the humoral immune response induced by various vaccines. On 0 day post vaccination (dpv), the geometric mean titers of maternal antibodies in all the groups were in the range from 4.5±0.52 to 5.3±0.54. The antibody titers started to rise non-significantly ($p<0.05$) in all the groups on 5th dpv although birds in some groups showed higher antibody titers A: 9.8±0.54 compared to others (B: 8.0±0.66, C: 7.5±0.87, D: 7.0±0.91and E: 3.1±0.64). The same trend of development of higher antibody titers with time was noticed on subsequent testing (10th dpv) with some non-significant variations. Birds of group A vaccinated with Ivac showed highest antibody titer (16±0.81) followed by groups C (Spvac) and B (Lvac) respectively. Similar trends of rising antibody titers were evidenced on follow up testing on 15th (from 24.3± 0.84 to 27.9 ±0.88) and 20th dpv (from 48.5 ± 0.54 to 55.7± 0.54). The birds were challenged on 25th day of age. The pattern of antibody titers declining was observed PCI (25th days of age) in all the vaccinated groups in comparison with control groups. On 5 days PCI (30 days of age), the geometric mean antibody titers showed declining trends in all the vaccinated groups ranging from 22.6 ± 1.24 to 27.9 ± 0.79 followed by an increasing trends in the antibody titers on subsequent testing on 10 day PCI (37.9±0.63 (B), 35.0±0.66(A), 34.3±0.87(C). In this study, on 15 days PCI, again a declining trend was evidenced in all the vaccinated groups (34.3 ±0.87 to 37.9 ±0.63). Thought-out the study trial, it was noticed that the variation in antibody titers were found non-significant among all the vaccinated groups however a significant difference was observed when compared with D (PC) and E (NC) groups (Table III).

Table II. Viral load quantification in broiler birds immunized with different imported, local and self-prepared vaccine against H9N2 (AIV).

Groups	Days														
	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
A	ND	ND	ND	730	1600	1940	1600	1223	1070	930	750	470	143	75	ND
	ND	ND	ND	470	1330	1645	1278	620	325	177	73	ND	ND	ND	ND
C	ND	ND	ND	430	1020	1550	1330	700	430	232.8	112	ND	ND	ND	ND
D	ND	ND	4854	51189.2	28152224.4	662564.1	425636.5	116173	55482.2	2232.8	1723	758	700	470	235
E	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

A, imported vaccine; B, local vaccine; C, self prepared vaccine; D, positive control; E, negative control.

Table III. Post vaccination HI mean antibody titer (GMT) in broiler birds immunized with different imported, local and indigenous vaccine against H9N2 (AIV).

Groups	Days (Mean ± SD)							
	5	10	15	20	25	30	35	40
A	5.3±0.54	9.8±0.54	16±0.81	24.3±0.84	52.7±0.7	27.9±0.79	35.0±0.66	32.3±0.51
B	4.9±0.44	8.0±0.66	13.9±0.75	27.9±0.88	48.5±0.54	25.1±1.02	37.9±0.63	35.2±0.69
C	5.3±0.54	7.5±0.87	16.0±0.89	25.9±1.10	55.7±0.54	22.6±1.24	34.3±0.87	30.7±0.44
D (PC)	4.5±0.52	7.0±0.91	5.3±0.51	4.9±0.50	4.2±0.48	10.7±0.71	22.4±0.52	20.27±1.34
E(NC)	4.6±0.72	3.1±0.64	2.3±0.79	2.4±0.87	2.2±0.74	2.8±1.08	2.0±0.87	2.6±0.63

A, imported vaccine; B, local vaccine; C, self-prepared vaccine; D, positive control; E, negative control.

DISCUSSION

In many countries of the world, vaccination and strict biosecurity measures helped to control avian influenza H9 virus outbreaks. Since 1998, AIV H9 is endemic in Pakistan and resulted in significant economic losses in the form of drop in egg production, poor FCR and mortality oftenly due to secondary bacterial infections (Ayaz *et al*., 2017; Khalil *et al*., 2017). Every year, during low humidity and wheat harvesting period, outbreaks of AIV H9 are reported more frequently. There is always continuous discussion on selection and use of local and commercial vaccines to control or minimize the losses incurred due to AIV H9. A number of commercial and local vaccines are available but their efficacy data is limited to humoral immunity and challenge protection. Data about potential of various vaccines to stop/reduce virus shedding post virus exposure is not available. In the present study the efficacy of commercial local and imported vaccines (A, B) was compared with self-prepared oil based inactivated vaccine (C). In this study, comparative potential of vaccines in virus shedding reduction (rRT-PCR) and developing humoral immunity (HI test) post challenge infection was evaluated.

A good vaccine must be able to protect flocks from filed challenge and to stop/minimize virus shedding. In this study, effect of various vaccines on virus shedding was evaluated post challenge infection. Virus shedding pattern and load was quantified by real time PCR from 1-15 days PCI. In this study, virus shedding was started one day earlier (3rd day) in non-vaccinated but challenged group than in the vaccinated groups (4th day). These findings are in line with the results obtained in another study where suppression of virus shedding by inactivated vaccines was documented (Tavakkoli *et al*., 2011). The delay in shedding of virus in vaccinated groups indicated that presence of circulating specific antibodies had played their role in reduction of AIV challenged virus either by enhancement of phagocytosis, blocking the virus replication or complement mediated lysis. Maximum virus shedding in all the vaccinated groups was observed on 6th day PCI which is in concordance with earlier study findings where highest virus shedding load was evidenced on the same day (Mosleh *et al*., 2009). Our study findings showed some disagreement with results of a study on virus shedding post challenge infection, where shedding was noted on 2nd day after infection (Elfeil *et al*., 2018). This slight disagreement may be due to variation in the immune status of birds at the time of challenge infection or use of different vaccine etc. Virus shedding started to decrease on 7th day PCI in all the vaccinated groups irrespective of the local vaccines used and shedding stopped on 12th day PCI in local (Lvac) and self-prepared (Spvac) vaccine groups (B and C) whereas in imported vaccine group (A) it continued and stopped completely on 15th day PCI. However, virus shedding continued throughout trial (15 days PCI) in the control group (D). Immune cell process virus, the pathogenicity of virus is decreased and vulnerability of host for disease is reduced (Germeraad *et al*., 2019). In the non-vaccinated challenged birds, viral load was found higher as compared to vaccinated groups throughout the trial. These findings were in concordance with the results reported earlier where higher virus load was evidenced in the non-vaccinated group (Tavakkoli *et al*., 2011). The possible reason of this higher viral load in non-vaccinated group is probably due to absence of specific immunity that neutralize the virus and cause decline in virus replication and then virus shedding. These findings depicted effectiveness of avian influenza vaccines in clearance of AI virus by local vaccines on 12 days PCI which is supported by another study results where shedding of virus stopped on 12 days PCI (Elfeil *et al*., 2018). In the imported vaccine group shedding completely stopped on 15th day PCI. Variation in the cession of shedding might be due to difference in the viral genome of vaccinal and challenged strain.

In current study no significant difference in local and self-prepared vaccine observed in virus shedding however

this difference was significant when compared data of local vaccines with imported vaccine. This difference can be due to the fact that there was 100% similarity in the viral genome of vaccinal (local) and challenged virus whereas shedding persisted longer in imported vaccine this can be due to the possible reason that vaccine and challenge strain was mismatched. Another study highlighted that if the vaccine and challenge strain are similar there will be maximum protection, if there is mismatch among vaccinal and field strain then shedding will be lasted for longer (Kang *et al*., 2020).

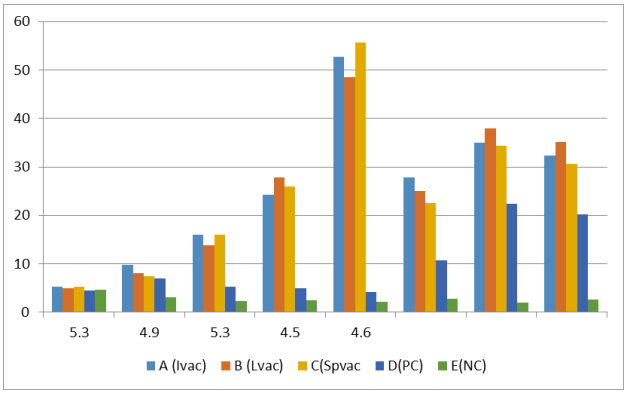

Fig. 1. Comparison of anti H9 antibody titers (GMT) in various vaccines groups post challenge infection.

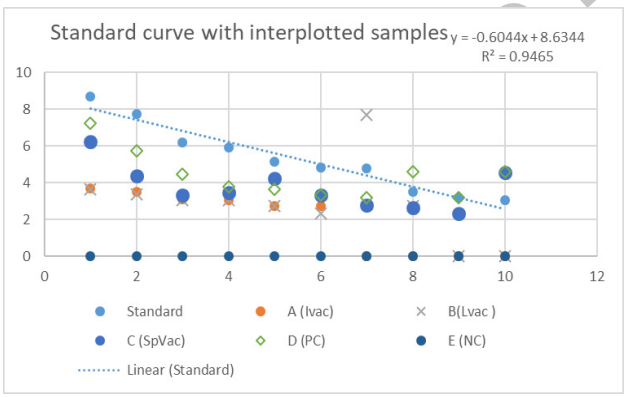

Fig. 2. Shedding of AIV H9 in different groups expressed in log 10 copies.

When data was analyzed statistically there was no significant difference (P>0.05) among local vaccine (Lvac and Spvac) groups in virus shedding but significant difference (P<0.05) observed when compared data of imported vaccine with local (Lvac and Spvac) vaccines. However, significant difference observed of all the vaccines either local or imported observed when compared data with control groups (D and E). These results disclosed that all the vaccines either local, self -prepared or imported vaccines are efficient to suppress virus shedding and replication in broiler birds.

Post vaccination H9 antibody response of different groups was measured. Detectable antibody titer was noticed in all the vaccine groups by the 1st week post vaccination (Table III). Gradual increasing antibody titer trend with respect to time was observed in all the vaccine groups except non-vaccinated control groups. The gradual increase in antibody titers is due to the processing and presentation of antigen by antigen presenting cells (APCs) along-with MHC-II to helper T cells which help B cell to differentiate into plasma cell leading to production of specific antibodies. These findings are supported by another previous study where gradual increase in antibody titers with respect to time has been reported (Richard-Mazet *et al*., 2014). It was also noticed that all the trial vaccines took almost 3 weeks to develop maximum level of antibodies which are in line with the findings of an earlier study in which maximum immune response was observed on 21 dpv (Shaukat *et al*., 2016). Interestingly a decline in antibody titer was noticed in all the vaccinated groups post challenge infection that was in concordance with the (Reham *et al*., 2019). The decreasing trend in antibody level PCI was persisted only for few days (5 days) and then started increasing. The decrease in antibody titer after challenge with the virus is probably due to the neutralization of antibodies and returning to increasing trend is the results of enhanced phagocytosis due to opsonization. All the vaccinated birds showed 100 percent protection against the challenge infection. No clinical signs were observed in vaccinated birds while mild respiratory signs were noticed in positive control group. Our findings are in agreement with the results reported by Sayed *et al*., where only mild clinical signs were observed without any mortality post AIV H9 challenge infection (Sayed *et al*., 2019). These findings depicts the circulation of probably low path AIV H9 virus in the field. However, our findings differ from results of other studies where AIV H9 caused high mortality with pronounced clinical signs (Parvin *et al*., 2015; Al-Garib *et al*., 2016; Houadfi *et al*., 2016). This disagreement may be due to circulation of high path AIV H9 viruses in their study areas.

This study declared that all the vaccines were effective in the reduction of viral shedding. Not only vaccine is needed for the control of deadly AIV but their focus should be on use of potent vaccine that is capable of inducing early post vaccination immune response (Talat *et al*., 2020). Various factors are involved in the vaccine efficacy i.e., vaccination time, dose of antigen in the vaccine. Our studied results declare that vaccination at the 1st week of birds' age was effective in the disease

development. Another study supported our results that vaccination at 7-14 days of life is suitable for the development of protective titer (Reham *et al*., 2019). This can be concluded that protective immune response can be achieved by the use of inactivated vaccines either local (commercial or self-prepared) or imported but there will be more protection if there is match among vaccinal and field virus. These results highlighted that vaccine strain should be regularly updated and evaluated to achieve optimal protection against the prevalent subtype and minimize economic loss.

Taken together, our results support previous findings that vaccines better matched to field isolates provide increased protection and decrease the risk of transmission by limiting virus shedding.

CONCLUSION

This study indicated that viral shedding evaluation is critical for determining the efficacy of avian influenza (H9) vaccines in broiler chickens. In this study none of the vaccine tested effective to completely stop the virus shedding in spite of inducing protective level of humoral immunity so effectors must be made to develop a vaccine which can stop the shedding of virus post exposure to the field virus to minimize the spread of disease.

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IRB approval

This study was approved by Institutional Review Board Committee of University of Veterinary and Animal Sciences, Pakistan (DAS: 1075).

Ethical approval

The study was carried out in compliance with guidelines issued by ethical review board and institutional biosafety committee of University of veterinary and Animal Sciences, Pakistan. The official letter (no. DR/35) would be available on fair request to corresponding author.

Statement of conflict of interest

The authors have declared no conflict of interest.

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