

## TRANSMISSIBILITY OF H9 SUBTYPE OF AVIAN INFLUENZA VIRUS IN VACCINATED AND NON-VACCINATED BROILER CHICKEN EXPOSED TO EXPERIMENTALLY CHALLENGED BIRDS

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### ABSTRACT

This study aimed to evaluate the efficacy of various avian influenza virus subtype H9 vaccines for their potential to stop or reduce the virus shedding and transmission in broiler birds exposed to challenge birds. At 6<sup>th</sup> day of age 3 groups of birds (A, B, C) comprising 10 birds each were vaccinated with imported (Ivac), local (Lvac) and self-prepared (Spvac) vaccines while groups D, E and F were kept as non-vaccinated control groups. On 25<sup>th</sup> day of age, birds of group D were challenged through intranasal route with 100 EID<sub>50</sub> of H9 virus and housed with vaccinated birds of groups A, B, C and non-vaccinated group E. Group F was kept in isolation in a separated shed. All the experimental birds were observed twice a day for any clinical signs of AI for 10 days and cloacal swabs were collected on daily basis, post exposure to evaluate the number of shedders and viral load quantification by real time RT-PCR. In directly challenged group D, virus shedding started one day earlier (2<sup>nd</sup> Day Post Challenge -DPC) than other groups (3<sup>rd</sup> Day Post Exposure-DPE). On 2 DPC, 50 % birds showed virus shedding with 3037.5copies/μL of viral load. In vaccines groups, 30-40 % shedders were identified with viral load ranging from 432.45 copies/μL on 3<sup>rd</sup> DPE. The number of virus shedders increased and reached maximum on 6<sup>th</sup> DPC (100%) with viral load 59427921.8 copies/μL copies in group D whereas the maximum shedders were evidenced on 6<sup>th</sup> DPE in all the vaccinated groups (50-60%) with viral load ranging from 2235.7copies/μL. The virus shedders and load were decreased in all the vaccine groups on 6<sup>th</sup> DPE. The virus shedding stopped on 9<sup>th</sup> DPE in local vaccinated group B than imported and self-prepared vaccinated groups (10<sup>th</sup> DPE) however in group D virus shedding didn't stop till the end of experimental trial. In group E, the number of shedders and viral load was less than group D but more than all the vaccinated groups throughout the experiment trail. None of the birds in group F showed virus shedding. Statistically all vaccines proved effective to reduce virus shedding and transmission than directly challenged group D and E (p<0.05) and may be used to combat AIV H9 infection. Based on this study it may be concluded evaluation of viral shedding is critical for determining the efficacy of avian influenza (H<sub>9</sub>) vaccines in broiler chickens

**Key Words:** Transmissibility, AIV-H9, Vaccination, Self-prepared Vaccine, Viral load Quantification

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### INTRODUCTION

In Pakistan, Livestock sector is growing day by day with 3.70 % growth rate and its contribution in national GDP is 11.5%. In Pakistan, poultry industry is the second largest industry that provides employment to more than 1.5 million people by direct or indirect ways (Economic Survey of Pakistan, 2020-2021). Worldwide, poultry industry is suffering from various bacterial and viral diseases. Among viral diseases, avian influenza virus (AIV) is hindering the progress of poultry industry due to high mortality, drop in egg production, and high medication cost (Aslam *et al.*, 2015)

Avian Influenza is a contagious viral disease that has been observed worldwide over the past hundred

years (Swayne *et al.*, 1999). Since 1994, various AIV outbreaks have been reported that resulted into either high mortality and morbidity or low mortality and high morbidity mainly in the unvaccinated poultry flocks in Pakistan (Guo *et al.*, 2000; Hasni *et al.*, 2021). AIV is an enveloped virus that belongs to *Orthomyxoviridae* family and classified into A, B and C genera on the basis of internal (NP and M1) proteins (Subtain *et al.*, 2011). Among these genera only influenza A viruses are classified into various subtypes based on hemagglutinin (HA) and neuraminidase (NA). To date, 18 HA and 11 NA subtypes have been documented (Shahzad *et al.*, 2020). On the basis of disease severity, AIV are categorized into low pathogenic (LPAIV) and high pathogenic (HPAIV) subtypes. HPAIV results into 100 %

mortality while LPAIV causes mild respiratory problems (Cui *et al.*, 2017).

In Pakistan , economic losses to the tune of around 2.2 billion rupees have been reported which emphasizes the need to control and eradicate AIV from poultry (Khan *et al.*, 2021). The low path AIV H9 subtype is endemic in Pakistan. Virus eradication from the poultry is considered impossible. Vaccination is considered a successful control measure that can reduce the risk of virus transmission by reducing virus shedding and transmission to susceptible flocks. Role of vaccine can be observed by limiting virus spread in the field, prevention of infection and minimizing virus replication in digestive and respiratory tract that control further viral transmission and disease outbreak (Swayne and Kapczynski, 2008).

In Pakistan, both local and imported AIV H9 vaccines are being used that claim to stop virus shedding in broiler birds but according to available literature there is no such supporting study findings to support the literature. Vaccines are being evaluated on the basis of development of humoral immunity but in-spite of having good level of antibodies; AIV H9 infections are continuously threatening the economy of poultry producers. Evaluation of virus shedding is critical to evaluate the efficacy of vaccines. This experiment was designed to evaluate the shedding and transmission pattern of virus in vaccinated and non-vaccinated birds exposed to challenged birds.

**MATERIALS AND METHODS**

This experiment was designed to evaluate the transmissibility of AIV (H9) from experimentally infected birds to vaccinated and non-vaccinated broiler birds.

**Virus inoculum stock:** 100 EID<sub>50</sub> of the low path H9 isolate (A/Chicken/Pakistan/UDL01/2019(H9N2) having accession no MN994294 was prepared and used in the experimental trial.

**Experimental Design:** A total of *n=60* one day old commercial broiler birds were purchased from Sabir’s

Hatchery Sheikhpura and divided into six groups (A, B, C, D, E and F) comprising 10 birds each. Cloacal swabs (*n=20*) collected randomly were tested for the absence of AIV H9 virus through RT-PCR. Two commercial (one local and imported each) and one self-prepared (Spvac) vaccines were used in the trial. Birds of groups A, B and C were vaccinated with imported (Ivac), local (Lvac) and self-prepared (Spvac) vaccines as per manufacturer instructions and PPA schedule. Birds of group D were challenged with 100EID<sub>50</sub> of the local isolate of AIV H9 (MN99429) through intranasal route on 25<sup>th</sup> day of age and kept with vaccinated (group A,B, C) and non-vaccinated birds (group E) to evaluate the transmissibility of virus. Group F was taken as negative control group and kept in isolation in a separate facility. The birds were examined every day up to 10 days post challenge infection to check the disease sign and symptoms in challenged and exposed birds.

**Evaluation of transmissibility of AIV H9 virus**

**Evaluation of virus shedders and quantitation of virus shedding through real-time RT PCR:** To evaluate the transmissibility of AIV H9 virus, cloacal swabs were collected at every 24 hours from all the groups post exposure to challenged birds to observe the number of virus shedders and quantitation of viral copies through real time reverse transcriptase PCR (q RT- PCR) using specific primers (HA gene) and probe (Table:1) as described earlier (Shabat *et al.*, 2010).Viral RNAs were extracted from cloacal swabs using QIA amp® nucleic acid extraction kit according to the manufacturer’s instructions. Reaction mixture was prepared in 25 µL final volume containing: 2 µL cDNA, 1 µL of each forward and reverse primer and 0.5 µL probe (H9 probe) (both having 20 pmol final concentration), 12.5 µL of 2xRT PCR Buffer and 7.5 µL of nuclease free water. The real time RT-PCR assay was run with the following cycling conditions: RT at 50°C for 30 mint and 95 °C for 15 minutes, followed by cycling steps of 40 cycles at 94 °C for 10 sec, and 60°C for 45 sec of PCR (Shabat *et al.*, 2010).

**Table 1. Real time RT-PCR primers and probe sequences**

Primer	Sequences
H9F	GGAAGAATTAATTATTATTGGTCGGTAC
H9R	GCCACCTTTTTTCAGTCTGACATT
H9Probe	FAM-5 -AACCAGGCCAGACATTGCGAGTAAGATCC-3-TAMRA

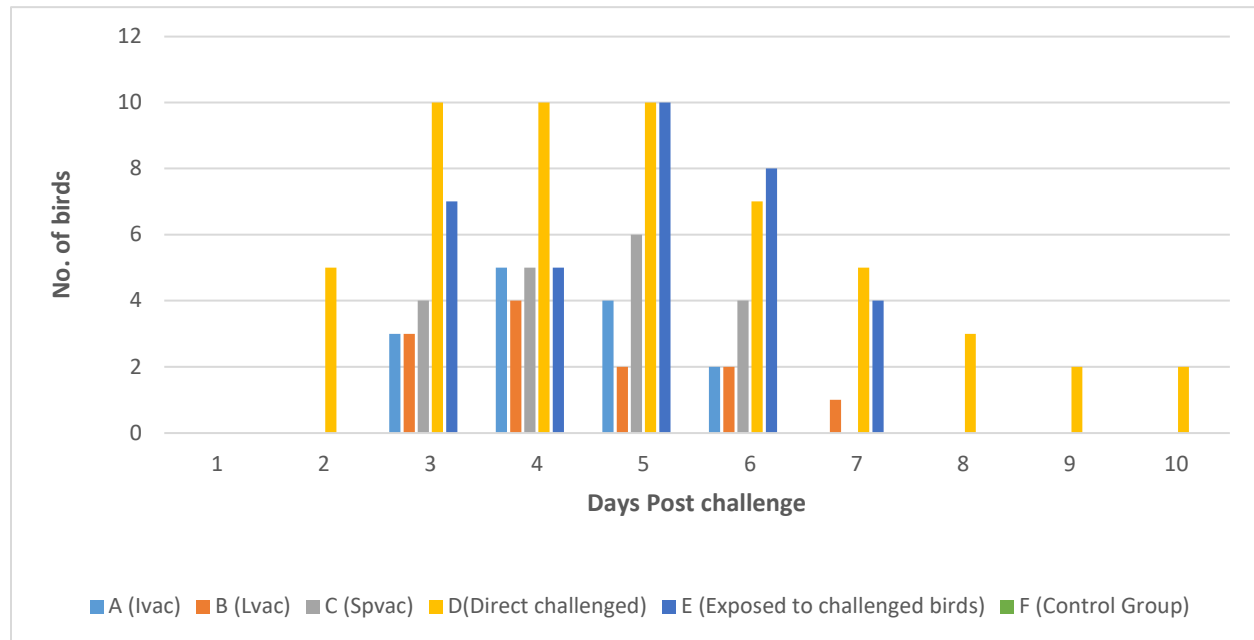
**Statistical Analysis:** The comparative potential of various vaccines to stop or reduce virus shedding was analyzed statistically using SPSS software by repeated measures ANOVA.

**RESULTS**

**Clinical signs and symptoms:** Mild clinical signs and symptoms were noted in all in-contact birds. Post

exposure to challenged birds, variation in clinical signs from apparent to mild respiratory distress were noted in all the experimental groups however number of birds exhibiting clinical signs of AIV H9 infection varied from group to group. All the birds (10/10) of group D that was challenged started showing clinical signs 2 day post challenge (DPC) and continued till the end of trial period. The group E that was non-vaccinated but kept with

challenged group D showed mild clinical signs in 5/10 (50%) birds. The clinical signs persisted for 5 days in exposed to challenged birds. In the vaccinated groups (imported or local), only 2 bird in each group showed mild clinical signs and continued till 5 days. In the non-vaccinated groups F that was not exposed to challenged group did not show any clinical signs (Figure 1).

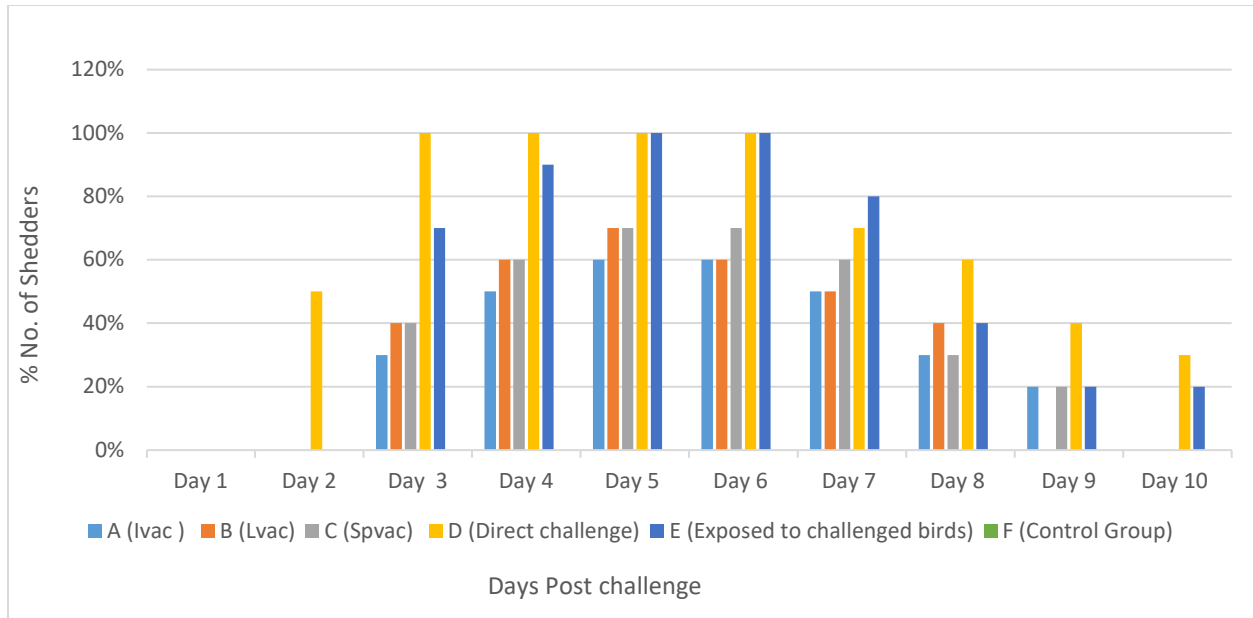


**Figure 1. Number of birds showing Clinical signs of AI V H9 post challenge Infection.**

**Evaluation of virus shedders:** To quantify the number of virus shedders in each experimental group, cloacal swabs collected on each day from 1-10 were tested through real time RT PCR. On 1<sup>st</sup> and 2<sup>nd</sup> DPC, none of the group showed virus shedding except directly challenged group D where 50% virus shedders were detected. The number of virus shedders raised up to 100% from 3<sup>rd</sup> to 6<sup>th</sup> DPC following decreasing trend on 7<sup>th</sup> (70%), 8<sup>th</sup> (60%), 9<sup>th</sup> (40%) and 10<sup>th</sup> (30%) DPC respectively. In all other in-contact groups, virus shedding started on 3DPC irrespective of vaccination status however number of virus shedders varied from group to group.

Group E that was non-vaccinated, non-challenged but in-contact to challenged birds, virus shedding (70%) started on 3<sup>rd</sup> day post exposure (DPE). On 4<sup>th</sup>, 5<sup>th</sup> and 6<sup>th</sup> day; 90% and 100% birds showed virus shedding respectively. The 100% shedding pattern was observed only for two days followed by decreased shedding trend on 7<sup>th</sup> (80%), 8<sup>th</sup> (40%), 9<sup>th</sup> and 10<sup>th</sup> (20%) DPE respectively.

In all the vaccinated groups, shedding of virus was observed on 3<sup>rd</sup> DPE. None of the vaccines either local, imported or self-prepared proved effective to completely stop the virus shedding. Slight variation in virus shedding among various vaccine experimental groups was noted. On 3<sup>rd</sup> DPE group A (Ivac) showed 30% virus shedders where as 40% shedders were observed in both locally prepared commercial vaccine group B (Lvacc) and self-prepared vaccine group C (Spvac). On 6<sup>th</sup> DPE, maximum number of virus shedders 60% in (Ivac and Lvacc) and 70% (Spvac) were observed. The number of virus shedders decreased on 7<sup>th</sup>, 8<sup>th</sup> and 9<sup>th</sup> (50%, 30% and 20%) DPE in A (50%, 40% and 0%) B and (60%, 30% and 20%) C groups respectively. On 9<sup>th</sup> DPE, the group B that was vaccinated with local commercially prepared vaccine, no virus shedder was detected however in other two vaccinated groups (A-Ivac, C-Spvacc), still virus shedders were observed. On 10<sup>th</sup> DPE no virus shedding was evidenced in group A and C (Figure 2).



**Figure 2. Number of shedders post exposure to AIV H9 infected birds**

**Quantitation of Viral load through real time PCR (qRT-PCR):** Real time RT-PCR was performed to quantify the shedding of H9 subtype of avian influenza virus by measuring the copies of RNA from cloacal swabs taken from birds of all experimental groups post exposure to challenged birds with respect to time. In the direct challenged group (D), 3037 RNA copies was detected on 2<sup>nd</sup> DPC. The maximum load (59427921.8 copies/ $\mu$ L) was detected on 5<sup>th</sup> DPC followed by decline on subsequent days (Table 2) throughout the experimental trial (10 DPC). Virus shedding was delayed by one day in all the vaccinated groups kept with challenged birds (group D). Among the vaccines groups,

slightly more RNA copies were detected in group B (590 copies/ $\mu$ L) than groups A (517.4 copies/ $\mu$ L) and C (432.45 copies/ $\mu$ L) on 3 DPE, however these copies were significantly less compared to that detected in group E (1594.05copies/ $\mu$ L). An increase in the level of RNA copies was observed in all the vaccine and exposed control (E) groups on follow up testing up to 6<sup>th</sup> DPE whereas in direct challenged group D, increasing trend was noticed up to 5<sup>th</sup> DPC. In the group F, none of the bird showed any virus shedding throughout the experimental period. Day wise results of all the groups have been mentioned in table 2.

**Table.2. Quantitation of viral load in vaccinated birds housed with challenged birds (non-vaccinated) at different days post infection**

Groups	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
<b>A</b>	ND	ND	517.4	1255.8	2517.6	2967	1105.6	573.4	230	ND
<b>B</b>	ND	ND	590	1272.8	2473.5	3122.2	1339.3	563.7	ND	ND
<b>C</b>	ND	ND	432.45	1452.4	2235.7	3173.5	1091.5	510	34	ND
<b>D</b>	ND	3037.5	62977	1619378	59427921.8	868892	157640	64767	3236.45	2199.5
<b>E</b>	ND	ND	1594.1	42880.8	28828.3	551624.8	6503	4438.75	2185	1430.75
<b>F</b>	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

A-Ivac , B-Lvac, C-Spvac, D-,Directly challenged, E- Exposed to challenged , F-Negative control

**DISCUSSION**

Avian influenza is devastating disease of poultry due to high economic losses and worldwide distribution (Shah *et al.*, 2014). In Pakistan AIV subtype of H9 is endemic since 1998 and is considered as primary gate for secondary bacterial infections (Ayaz *et al.*, 2017; Taha *et*

*al.*, 2019). Co infection with other pathogens in the poultry farms leads to the complications and misdiagnosis of disease (Costa-Hurtado *et al.*, 2014). AIV H9 is low pathogenic but still it results in significant economic losses due to low productivity, poor growth rate and high medication cost while in the layers it results in drop in egg production (Shaukat *et al.*, 2016; Sultan *et*

*al.*, 2017). AI can best be controlled by preventing the introduction of virus in the field. Vaccines provide effective way to control the disease (Shah *et al.*, 2014). An effective vaccine can stop/slow down the spread of virus by decreasing the shedding of the virus.

In Pakistan, a number of local and imported avian influenza inactivated vaccines are being used with the claim to stop/decrease the virus shedding and spreading, however as per available literature there is no such study findings. This experiment was therefore designed to study the shedding and transmission pattern of virus in vaccinated birds in comparison to non-vaccinated birds exposed to intentionally AIV H9 exposed birds. For this purpose, two commercial (Ivac, Lvac) and self-prepared (Spvac) vaccines were evaluated. Birds were vaccinated as per manufacturer's protocol and PPA schedule. The clinical signs, mortality, virus shedding and transmission pattern were observed.

In our transmission experiment, observation of mild clinical signs including slight respiratory rales in all the groups were accepted as indication of virus transmission. These observations are in line with findings of a previous study where mild clinical signs were observed post exposure to the virus (Alexander, 2007; Elfeil *et al.*, 2018). However, our findings were not in agreement with the results of another study, where severe clinical signs and mortality was reported post exposure to challenge infection. This disagreement may be due to the variation in the pathotype of AI virus, co infection with any other pathogen or un detected field conditions (Hablolvarid *et al.*, 2013).

To evaluate the efficacy of vaccines for reducing/ stopping the virus transmission and shedding pattern of virus from intentionally challenged to exposed birds, cloacal swabs were collected and tested. In the study virus shedders were counted in each group followed by quantitation of viral load by real time RT-PCR. Avian influenza virus (AIV) shedding in vaccinated birds exposed to infection occurs when the virus is excreted from the infected bird primarily through nasal and oral secretions, or through feces (Kilany *et al.*, 2016; Talat *et al.*, 2020). In our hands, none of the vaccine was found effective to stop the virus shedding although the number of shedders were significantly less than non-vaccinated groups which is in concordance with the results of a previous study where vaccines did not stop the virus shedding (Cui *et al.*, 2021). These findings indicated that in vaccinated birds, the AIV may still replicate in the respiratory and intestinal tracts, although at a reduced level compared to unvaccinated birds. As a result, vaccinated birds can still shed the virus even if they do not exhibit severe clinical signs of the disease (Capua *et al.*, 2004; Channa *et al.*, 2021)). However, none of the negative control bird exhibited clinical signs and shedder which are in line with the findings of Belkasmi *et al.* where similar pattern of AIV was

observed till the end of the experiment (Belkasmi *et al.*, 2020). When the various trial vaccines were compared with respect to their potential to reduce the number of virus shedders, it was noticed, that maximum number of shedders were observed in self-prepared vaccinated group compared to local and imported vaccinated groups however statistically the variation was non-significant. This non-significant disagreement may be due to variation in the strain of avian influenza virus

In this study, when the time of virus shedding post challenge infection and exposure to challenged birds was studied, virus shedding started one day earlier in the directly challenged group (2<sup>nd</sup> DPC) compared to all the vaccinated and non-vaccinated groups (3<sup>rd</sup> DPE), indicated the potential of vaccines to delay the virus shedding. This could be due to the fact that in non-vaccinated groups, the immune system of the birds has not been primed to recognize and respond to the avian influenza virus, which means that the virus can replicate more quickly and cause more severe disease. This can lead to earlier shedding of the virus, as the infected birds produce more virus particles that can be released into the environment through their respiratory secretions, feces, and other bodily fluids. On the other hand, in vaccinated groups, the birds have been vaccinated with a vaccine that contains inactivated avian influenza virus. This primes the birds' immune systems to recognize and respond to the virus, so that if they are infected, their immune system can quickly mount a response to control the virus and limit its replication. As a result, vaccinated birds may shed the virus for a shorter period of time and at lower levels compared to non-vaccinated birds, as their immune system is able to control the infection more efficiently (Jang *et al.*, 2018; Van der Goot *et al.*, 2005). In our hands, the level of RNA copies in feces of birds quantified throughout the trial period was proportional to the number of shedders with respect to time. Highest level of RNA was detected on 5<sup>th</sup> DPC in the directly challenged group whereas it was on 6<sup>th</sup> DPE in all the vaccines and exposed group E.

In the present study less number of viral copies was observed in all the vaccinated groups than in non-vaccinated directly challenged and non-vaccinated exposed group. This is due to the reason that vaccinated bird after exposure to the virus, quickly recognizes the virus and begins to mount a response to control and eliminate it. This response involves the production of specific antibodies that can bind to the virus and prevent it from infecting other cells, as well as the activation of immune cells such as T cells and natural killer cells that can kill infected cells and limit the spread of the virus. By controlling the replication and spread of the virus, the immune response can reduce the amount of virus that is shed from the bird's body. This can result in lower viral loads in the feces and other bodily fluids of vaccinated birds compared to non-vaccinated birds, which can in

turn reduce the risk of transmission to other birds and animals (Chen *et al.*, 2022).

Higher level of viral RNA copies in control groups (D, E), showed the high replication of virus in the GIT as reported in another study (Mosleh *et al.*, 2009). From 6<sup>th</sup> DPE onward, number of shedders, the viral RNA copies were also decreased in all the groups. This declining trend in experimental birds was probably due to development of immunity with respect to time leading to elimination of virus (Kilany *et al.*, 2016). These findings are in line with our studied results as there was reduced virus shedding and transmission in the vaccinated birds from infected birds. Vaccination is considered to reduce shedding but unfortunately it does not help in the prevention of infection. In this study, there was non-significant difference among all the vaccines regarding their potential to reduce/stop the virus shedding/spreading and viral load excreted post exposure to the challenged group however the difference was significant when compared with directly challenged group.

**Conclusion:** On the basis of these study findings it could be concluded that avian influenza virus vaccines are effective in reducing viral shedding and transmission of the virus and should be used in combination with other control measures such as biosecurity and regular surveillance, to effectively control the spread of avian influenza virus.

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