Project ID: 694

Competitive Research Grant

Sub-Project Completion Report

on

Assessment of effectiveness of avian influenza vaccination in commercial layer birds in Bangladesh

Project Duration

April 2017 to September 2018

Department of Medicine, Bangladesh Agricultural University, Mymensingh Bangladesh

Submitted to
Project Implementation Unit-BARC, NATP 2
Bangladesh Agricultural Research Council
Farmgate, Dhaka-1215

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Project Implementation Unit
National Agricultural Technology Program-Phase II Project (NATP-2)
Bangladesh Agricultural Research Council (BARC)
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Published in:

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Acronyms

Ab	:	Antibody
AI	:	Avian Influenza
AIV	:	Avian Influenza Virus
ANOVA	:	Analysis of Variance
cELISA	:	Competitive ELISA
CV	:	Coefficient of Variation
DOC	:	Day old chick
ELISA	:	Enzyme Linked Immunosorbent Assay
HA	:	Haemagglutinin
HPAI	:	Highly Pathogenic Avian Influenza
HVT	:	Herpes Virus of Turkey
IM	:	Intramuscular
OD	:	Optical Density
PI	:	Percent Inhibition
qPCR	:	Quantitative Polymerase Chain Reaction
S/P ratio	:	Sample to positive ratio
SC	:	Subcutaneous
SD	:	Standard Deviation
SE	:	Standard Error

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Executive Summary

In Bangladesh poultry vaccination against avian influenza (AI) was initiated experimentally in 2012 with two vaccines, Re-6 and HVT-AIV. Since initiation of AI vaccination in Bangladesh, evaluation of the immunity level in poultry has not been performed so far. Therefore, a longitudinal study was undertaken to assess the immune status of the vaccinated birds against AI along with assessment of immune escaped AI virus (AIV) shedding in AI vaccinated flocks. A total of 720 sera samples, collected at different occasions from both vaccinated and unvaccinated flocks in Bogura, Joypurhat, Kishoreganj and Gazipur districts, were subjected to enzyme linked immunosorbent assay to determine antibody titre. Some selected cloacal swabs were subjected to Real time PCR to evaluate virus shedding.

Irrespective of area and farms the mean ELISA antibody titre of birds vaccinated with Re-6 vaccine was significantly ($p<0.01$) higher than the titre of HVT-AIV vaccinated and the unvaccinated birds at all the three occasions (3, 9 and 15-week post-vaccination). Among 480 birds vaccinated with either of the two vaccines (HVT-AIV and Re-6), 218 (45.4%) birds found to have positive ELISA antibody titre against AIV Type A. The overall percentage of positive birds was higher (74.2%) for Re-6 vaccinated flocks than HVT-AIV vaccinated flocks (16.7%), and the percentage of positive reactors increased over time at 3-, 9-, and 15-week post vaccination. Among the Re-6 vaccinated birds, 63.7%, 76.3% and 82.5% birds found to have positive ELISA antibody titre at 3-, 9-, and 15-week post vaccination, respectively, while 2.5%, 15% and 32.5% were positive among HVT-AIV vaccinated birds at the same time point. Re-6 vaccine produced protective and consistent humoral Ab titre against AIV type A in farms from Gazipur district those had good biosecurity compared to farms of Kishoreganj district, which had moderate to poor biosecurity. HVT-AIV vaccine failed to produce protective humoral Ab titre against AIV type A. Positive H9 titre was found in 21% vaccinated birds though H9 vaccine was not practiced in the studied flocks. It was also noticed that around 16% of the AI vaccinated birds shed virus in the droppings that may facilitate further spread of the virus. The information generated from the study will be helpful for poultry raisers, the ultimate beneficiaries, in selecting the effective vaccine to protect their birds from AI, and also for the policy makers in planning the strategic vaccination program with AI vaccines.

CRG Sub-Project Completion Report (PCR)

A. Sub-project Description

1. **Title of the CRG sub-project:**
   Assessment of effectiveness of avian influenza vaccination in commercial layer birds in Bangladesh

2. **Implementing organization:**
   Department of Medicine, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh

3. **Name and full address with phone, cell and E-mail of PI/Co-PI (s):**
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   Bangladesh Livestock Research Institute (BLRI), Savar, Dhaka-1341
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4. **Sub-project budget (Tk):**
   4.1 Total: Tk. 26,14,375.00
   4.2 Revised (if any):

5. **Duration of the sub-project:**
   5.1 Start date (based on LoA signed): April 2017
   5.2 End date : September 2018

6. **Justification of undertaking the sub-project:**

Highly pathogenic avian influenza (HPAI) is a deadly disease of poultry; also has zoonotic implication. Vaccines are used in integrated control strategies to protect poultry against H5N1 highly pathogenic avian influenza (HPAI) infection (Swayne et al., 2015). The disease H5N1 HPAI was first reported in Bangladesh in March, 2007 (Alam et al., 2010) and since then there have been over 556 reported outbreaks of HPAI in poultry, of which the majority have been handled using stamping-out (culling) strategies for control. Over 15 countries have publicly utilized poultry vaccination as a control tool to augment stamping out when the HPAI virus became endemic (Swayne et al., 2011). Likewise, in Bangladesh, poultry vaccination against AI was initiated experimentally in 2012 (http://www.thedailystar.net/news-detail-237244) and since then vaccination programs have been taken in so far nine districts of the country such as Dhaka, Gazipur, Kishoreganj, Narsingdi, Narayanganj, Tangail, Bogra, Joypurhat and Chittagong. Presently, Re-6 and HVT-AIV vaccines are being used in Bangladesh. Re-6 is an inactivated H5N1 AI vaccine that bears the HA and NA genes from a clade 2.3.2.1 H5N1 virus. HVT-AIV is a recombinant AI vaccine, which contains the

hemagglutinin (HA) gene of an H5N1 AIV and the herpes virus of turkeys (HVT) as the vector. Experimental studies have shown that various vaccines (either heterologous or homologous) are able to induce good clinical protection against a challenge infection with H5N1 strains of HPAI (Kumar et al., 2007; Swayne, 2009).

The minimum onset of protective immunity conferred by an inactivated vaccine begins at 2-week post-vaccination (Sims, 2007) and could last up to 1 year post-immunization (Tian et al., 2005). Hsu et al. (2010) found 92-94% efficacy for homologous inactivated vaccines and 68-71% effective for heterologous inactivated avian influenza vaccines in chicken. It has been reported that recombinant vaccines are also able to confer protection after single dose (Swayne et al., 1997; Qiao et al., 2006). However, inactivated vaccines provided superior protection against the H5N8 and H5N2 viruses in a single vaccination strategy compared with the recombinant vaccines (Kapczynski et al., 2017). The type and quality of vaccine, vaccination schedule, the dose and method of administration are the factors that can influence vaccination outcome (Tarigan et al., 2018). The efficacy may vary depending on the challenge clade, which highlights the need for continuous assessment against emergent HPAI/H5N1 (Villanueva-Cabezas et al., 2017). The effectiveness of vaccination carried out under field conditions is often lower than controlled laboratory conditions (Bouma et al., 2009; Poetri et al., 2014). It is, however, questionable whether the application of vaccines will induce clinical protection for poultry but will facilitate silent spread of the infection and human exposure to the virus, as by mid-2005, reports of vaccine failures emerged from the field in Indonesia (Bouma et al., 2008). Therefore, virus shedding would be an important biosecurity issue for further AI endemic control (Hsu et al., 2010). The virus shedding can be assessed and quantified by a one-tube hydrolysis fluorescent probe based real-time RT-PCR (RRT-PCR), which is specific, sensitive, easy to perform, and rapid, will be useful for virological, pathogenesis, and protection studies (Lee and Suarez, 2004).

Since initiation of AI vaccination in Bangladesh, evaluation of the immunity level in poultry has not been performed so far. Despite using vaccines against AI not much is known about the efficacy of vaccination and a quantitative assessment of effectiveness under field conditions has not been carried out in Bangladesh. The aim of the research was to answer the question: “what is the efficacy of commercial vaccine formulations administered to healthy commercial layer birds in Bangladesh against HPAI in terms of immunogenicity?” that will be helpful in strategic AI vaccination programme in Bangladesh. Furthermore, the quantification of virus shed from infected birds is valuable in pathogenesis studies and to determine the effectiveness of vaccines (Lee and Suarez, 2004). Therefore, the proposed research was also aimed at quantification of AIV to assess the shedding of viruses in vaccinated flocks.

7. Sub-project goal:

The ultimate goal is the containment of avian influenza (AI) in commercial layer birds in Bangladesh

8. Sub-project objective (s):

a) To measure the antibody titre against AIV Type A in commercial layer birds
b) To determine subtype (H5, H9, N1) specific immune response through cELISA
c) To assess the immune escaped AI virus (AIV) shedding in AI vaccinated flocks

9. Implementing location (s):

i. Department of Medicine, Bangladesh Agricultural University, Mymensingh
ii. NRL-AI, Animal Health Research Division, BLRI, Savar, Dhaka

10. Methodology in brief:

10.1. Measuring the antibody titre against AIV Type A in commercial layer birds

Study areas and design

A longitudinal observational study was carried out in 24 commercial layer poultry farms (16 AIV vaccinated and 8 unvaccinated). Farms were selected randomly following a multistage sampling technique: four districts from nine districts (Dhaka, Gazipur, Kishoreganj, Narsingdi, Narayanganj, Tangail, Bogra, Joypurhat and Chattagram) where vaccination of poultry against avian influenza are being practiced, two upazilas from each district, 3 farms (2 vaccinated and 1 unvaccinated) from each upazila (Table 1). Unvaccinated farms proximate to the vaccinated farm were selected for sample collection. List of the poultry farms was obtained from the respective Upazila Livestock Office as well as through personal communication with the feed and drug sellers.

Table 1: List of selected districts and upazilas

SN	Name of District	Name of Upazila
1.	Bogura	Bogura Sadar, Shahjahanpur
2.	Kishoreganj	Kishoreganj Sadar, Bazitpur
3.	Joypurhat	Kalai, Joypurhat Sadar
4.	Gazipur	Sreepur, Gazipur Sadar

Collection of flock data and samples

A pretested structured questionnaire was used to collect flock data including overall flock performance, disease and treatment history, information about previous AI outbreak, vaccination program, and status of AI vaccination as well as biosecurity of the farm. Prior to collection of data and sample a written consent was taken from the farm owner. Blood (sera) and cloacal swabs were collected for three occasions from each flock (Figure 1-2). The interval of sample collection was dependent on the status of vaccination. The 1st sampling was done at least 3 weeks after vaccination and then 2nd sampling was done at 9th week post vaccination and 3rd sampling at 15th week post vaccination. Blood samples from 10 birds and cloacal swabs from 5 birds from each farm on each occasion were collected. All the samples were kept at 4°C and transferred to the laboratory as soon as possible. A total number of 720 sera samples and 360 cloacal swabs were collected throughout the study period.

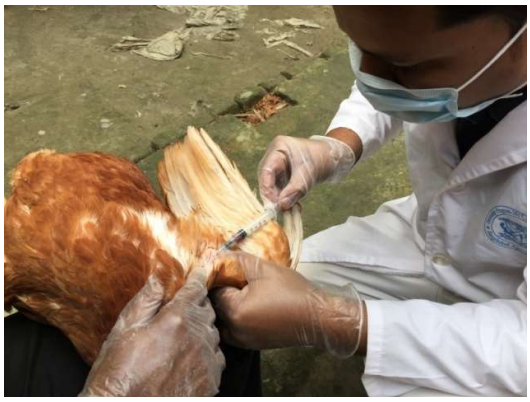

Figure 1. Blood collection from layer birds

Figure 2. Serum samples after separation

Vaccination Summary

Avian influenza vaccination is being practiced in Bangladesh since 2012. Currently two market preparations of avian influenza vaccine are found to be administerd in commercial layer birds. HVT-AIV, a recombinant vector vaccine used most commonly in Sonali layer breed in Bogura and Joypurhat whereas Re-6, an inactivated H5N1 vaccine was used in Kishoreganj and Gazipur districts. The common vaccination protocol for HVT-AIV was practiced in all the farms in Bogura and Joypurhat whereas different vaccination schedule was obserbed in Kishoreganj and Gazipur districts with Re-6 vaccine. In case of Re-6 according to the manufacturer's instruction a booster vaccination after 35 days of primary vaccination is required to attain protective immunity but no farm was found to administer a booster vaccination during the study period (Table 2).

Table 2. Vaccination summary of farms under the study

District	Upazila	Farm ID*	Vaccine**	Dose (mL)	Route	Age (Day) at Vaccination	
						1st	2nd
Bogura	Sadar	BS1	HVT-AIV	0.2	SC behind neck	DOC	-
		BS2					-
	Shahjahanpur	BSp1					-
		BSp2					-
Joypurhat	Sadar	JS1					-
		JS2					-
	Kalai	JK1					-
		JK2					-
Kishoreganj	Sadar	KS1	Re-6	0.5	SC behind neck /IM	11	-
		KS2				16	-
	Bazitpur	KB1				10	-
		KB2				17	-
Gazipur	Sadar	GS1				19	-
		GS2				24	-
	Sreepur	GSr1				16	-
		GSr2				39	-

** Farm ID used in order to maintain farms anonymity, derived from abbreviation for the district and upazila in which the farm was located. **HVT-AIV = Recombinant AI vaccine, which contains the hemagglutinin (H) gene of an H5N1 AIV and the herpes virus of turkeys (HVT) as the vector, Re-6 = Inactivated H5N1 vaccine. DOC = Day Old Chick*

Enzyme Linked Immunosorbent Assay (ELISA)

Indirect ELISA for detection of antibodies against Avian Influenza Type A

All the sera samples (n = 720) were subjected to Indirect ELISA using the commercial Influenza A ELISA Kit (Biocheck UK Ltd, UK) following the manufacturer's instruction (Figure 3). The kit is capable of detecting group specific antibody across all the H-types of AI virus. The S/P ratio (sample to positive ratio) and the antibody titer were calculated by using the following formulas.

$$\text{S/P ratio} = \frac{\text{Mean of test sample} - \text{Mea of negative control}}{\text{Mean of Positive control} - \text{Mean of Negative control}}$$

Antibody titer = Log_{10} Titer = 1.0 * Log (S/P) + 3.52

Antilog_{10} = Titer

Figure 3. Performing ELISA for detection of antibodies against Avian Influenza Type A

10.2. Determination of subtype (H5, H9, N1) specific immune response through cELISA

Competitive Enzyme Linked Immunosorbent Assay (cELISA) for detection of antibodies against H5 and H9 subtypes

Sera samples having positive titre for Influenza type A in indirect ELISA (n = 218) were subjected to cELISA using the commercial ELISA Kit for Avian Influenza virus H5, H9 antibody (Shenzen Lvshiyuan Biotechnology Co., Ltd.) following the manufacturer's instruction. The calculation of PI (blocking rate) and interpretation were made as below.

- PI (blocking rate) = (1 -Sample value/ Average value of Negative control) X 100%
- PI > 50% was judged as positive immunity and Pl < 50% was judged as no immunity

cELISA for detection of antibodies against N1 subtype

Sera samples having positive titre for influenza type A in indirect ELISA (n = 218) were also subjected to cELISA using the commercial ELISA kit (Sunlong Biotech Co., Ltd) for detection of antibodies against N1 subtype following the manufacturer's instruction. The critical value (CUT OFF) was calculated as Critical value = the average value of negative control + 0.15. If the OD value< CUT OFF, the sample was considered as AIV-N1 negative, and if the OD value >CUT OFF, the sample was considered as AIV-N 1 positive.

10.3. Assessment of the immune escaped AI virus (AIV) shedding in AI vaccinated flocks

Real-time RT-PCR (rRT-PCR)

A one-tube hydrolysis fluorescent probe based rRT-PCR with the cloacal swabs from both vaccinated (n = 98) and unvaccinated (n = 2) flocks were applied for the quantification of AI (Lee and Suarez, 2004). Genomic RNA was extracted from pooled samples using Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany), according to instructions from the manufacturer. rRT-PCR was performed with specific primer set for M gene of AIV from published literature (Spackman et al., 2002; Heine et al., 2007). AIV-M PCR positive samples were also screened by qPCR for AIV subtypes H5, N1 (Fig. 4). This was carried out in the National Reference Laboratory for Avian Influenza (NRL-AI), Animal Health Research Division, Bangladesh Livestock Research Institute (BLRI), Savar, Dhaka.

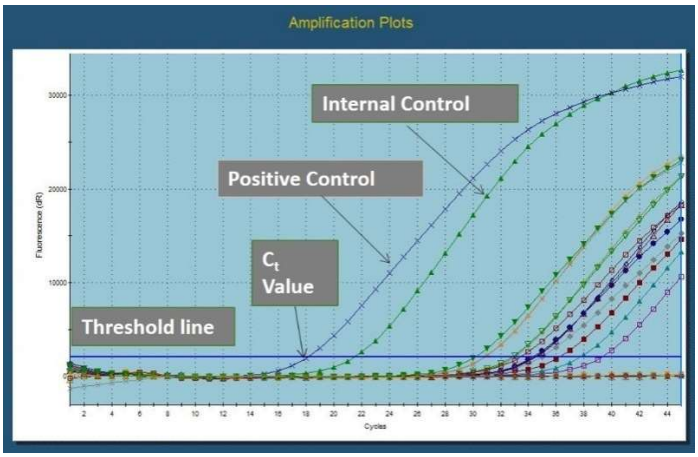

Figure 4. Real-time PCR for typing and sub-typing of AIV shedded in the droppings

Analysis of data

Repeated measures ANOVA was done to find out the significant difference in mean antibody titre of two different vaccines at different occasions using statistical package SPSS v22.0 for windows.

11. Results and discussion

11.1. Results

Measuring the antibody titre against AIV Type A in commercial layer birds and Determination of subtype (H5, H9, N1) specific immune response

Antibody titre and positive reactors against Avian Influenza type A

At 3- and 9-week post vaccination, none of the flocks, vaccinated with HVT-AIV vaccine in Bogura and Joypurhat districts, had mean positive antibody titre. However, at 15-week post vaccination, 5 flocks (out of 8), vaccinated with the same vaccine, had mean positive antibody titre, but the co-efficient of variation (CV%) was very large (89.6-113.8%) that indicates inconsistent level of titre in the flock. In Kishoreganj district, the mean positive titre was observed in 2, and 3 flocks (out of 4) at 9- and 15-week post vaccination, vaccinated with Re-6 though the CV% was above 40% (40.6%-74.5%), which indicates unsteady flock immunity. On the contrary, in Gazipur district, the mean ELISA antibody titre was positive in all the 4 flocks, vaccinated with Re-6, and the CV% of antibody titre in these flocks was 4.7%-22.4% that indicates the homogenous immune response in the flocks and very good flock immunity (Figure 5-8, Table 3).

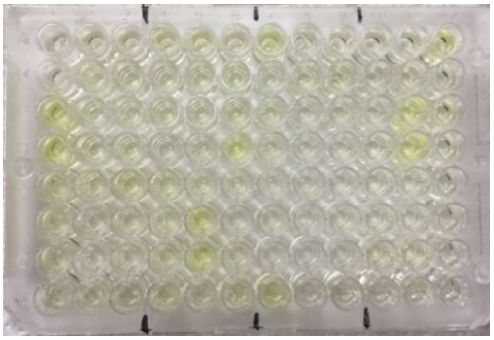

Figure 5. Results of ELISA for detection of antibodies against Avian Influenza Type A

Figure 6. Results of ELISA for detection of antibodies against Avian Influenza H5 subtype

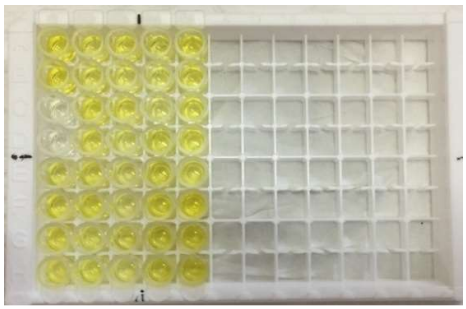

Figure 7. Results of ELISA for detection of antibodies against Avian Influenza H9 subtype

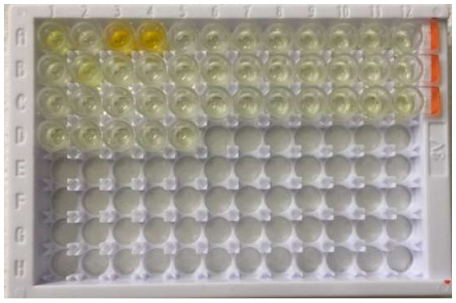

Figure 8. Results of cELISA for detection of antibodies against Avian Influenza N1 subtype

Irrespective of area and farms the mean ELISA antibody titre of birds vaccinated with Re-6 vaccine was significantly ($p<0.01$) higher than the titre of HVT-AIV vaccinated and the unvaccinated birds at all the three occasions (3, 9 and 15-week post-vaccination) (Figure 9). Similarly, district-wise comparison revealed that the mean ELISA Ab titre was very significantly higher in birds sampled from Gazipur district (Figure 10).

Among the 480 birds vaccinated with either of the two vaccines (HVT-AIV and Re-6), 218 (45.4%) birds found to have positive ELISA antibody titre against AIV Type A. One bird among 240 unvaccinated birds also had positive ELISA antibody titre. The overall percentage of positive birds was higher (74.2%) for Re-6 vaccinated flocks than HVT-AIV vaccinated flocks (16.7%), and the percentage of positive reactors increased over time at 3-, 9-, and 15-week post vaccination (Figure 11). Among the Re-6 vaccinated birds, 63.7%, 76.3% and 82.5% birds found to have positive ELISA antibody titre at 3-, 9-, and 15-week post vaccination, respectively, while 2.5%, 15% and 32.5% were positive among HVT-AIV vaccinated birds at the same time point. District-wise results revealed that the birds sampled from Bogura, Joypurhat and Kishoreganj were poor responder to AI vaccine (Figure 12). However, it is notable that all the vaccinated birds sampled from Gazipur district were positive responder to Re-6 vaccine at all the occasions (Figure 12). It is mentioned that overall biosecurity score was good in all the flocks of Gazipur district while in other districts the score was moderate to poor (Table 4).

Table 3. Farmwise antibody titre against Avian Influenza type A in layer birds of different vaccinated farms at different occasions in the selected districts

District	Upazila	Farm ID* (n = 10)	Vaccine used**	Post-vaccination ELISA Antibody titre Mean ± SE (CV%, NP)		
				3 Wk PV	9 Wk PV	15 Wk PV
Bogura	Sadar	BS1	HVT-AIV	156.8±22.3 (44.9, 0)	375.8±104.4 (87.8, 2)	434.2±99.1 (72.2, 3)
		BS2	HVT-AIV	148.2±24.6 (52.5, 0)	204.3±32.3 (50.0, 0)	676.5±224.1 (104.8, 3)
		BS3	Non-vaccinated	126.4±23.8 (59.4, 0)	141.5±18.7 (41.9, 0)	161.3±17.8 (34.9, 0)
	Shahjahanpur	BSp1	HVT-AIV	119.3±29.4 (77.9, 0)	360.6±127.1 (111.4, 2)	584.8±95.2 (51.5, 3)
		BSp2	HVT-AIV	174.3±88.2 (160.1, 1)	286.7±120.1 (132.4, 2)	962.9±346.4 (113.8, 3)
		BSp3	Non-vaccinated	51.5±16.2 (99.3, 0)	157.9±26.8 (53.7, 0)	146.4±30.4 (65.7, 0)

District	Upazila	Farm ID* (n = 10)	Vaccine used**	Post-vaccination ELISA Antibody titre Mean ± SE (CV%, NP)		
				3 Wk PV	9 Wk PV	15 Wk PV
Joypurhat	Sadar	JS1	HVT-AIV	177.0±27.1 (48.4, 0)	396.3±112.8 (90.0, 2)	419.9±132.1 (160.1, 2)
		JS2	HVT-AIV	156.7±25.7 (51.9, 0)	248.4±53.4 (68.0, 0)	721.2±247.6 (108.6, 3)
		JS3	Non-vaccinated	144.1±28.5 (62.5, 0)	148.6±19.0 (40.5, 0)	167.2±19.3 (36.5, 0)
	Kalai	JK1	HVT-AIV	291.3±51.9 (56.3, 0)	380.1±123.9 (103.1, 2)	962.9±299.9 (98.5, 4)
		JK2	HVT-AIV	185.3±89.8 (153.2, 1)	313.9±132.4 (133.4, 2)	1344.8±380.9 (89.6, 5)
		JK3	Non-vaccinated	131.8±54.2 (130.0, 0)	171.2±30.9 (57.1, 0)	203.7±23.3 (36.2, 0)
Kishoreganj	Sadar	KS1	Re-6	503.7±176.0 (110.5, 4)	859.7±121.9 (44.8, 7)	962.3±197.2 (64.8, 7)
		KS2	Re-6	534.3±154.2 (91.2, 5)	739.8±95.0 (40.6, 6)	875.8±115.6 (41.7, 7)
		KS3	Non-vaccinated	77.4±20.9 (85.4, 0)	259.1±35.6 (43.5, 0)	272.1±62.5 (72.6, 1)
	Bazitpur	KB1	Re-6	103.4±20.7 (63.2, 0)	403.0±94.0 (73.8, 3)	891.9±258.7 (91.7, 6)
		KB2	Re-6	262.0±107.8 (130.2, 2)	488.5±135.4 (87.7, 5)	657.2±154.8 (74.5, 6)
		KB3	Non-vaccinated	111.2±9.8 (27.8, 0)	175.4±24.6 (44.3, 0)	183.6±29.6 (50.9, 0)
Gazipur	Sadar	GS1	Re-6	7310.3±207.3 (9.0, 10)	7506±157.0 (6.6, 10)	9508.1±608.9 (20.3, 10)
		GS2	Re-6	7301.6±189.7 (8.2, 10)	7707.2±366.1 (15.0, 10)	7827.1±422.5 (17.1, 10)
		GS3	Non-vaccinated	44.8±14.2 (100.6, 0)	186.9±22.8 (38.7, 0)	306.7±33.0 (34.1, 0)
	Sreepur	GSr1	Re-6	6153.6±436.7 (22.4, 10)	8938.4±189.7 (6.7, 10)	9812.7±302.1 (9.7, 10)
		GSr2	Re-6	6043.8±372.5 (19.5, 10)	9101.6±134.0 (4.7, 10)	8418.3±354.7 (13.3, 10)
		GSr3	Non-vaccinated	91.2±7.8 (26.9, 0)	169.3±32.2 (60.1, 0)	166.9±33.9 (64.1, 0)

** Farm ID used in order to maintain farms anonymity, derived from abbreviation for the district and Upazila in which the farm was located, n = No. of birds sampled from each farm on each occasion.*

***HVT-AIV = Recombinant AI vaccine, which contains the hemagglutinin (H) gene of an H5N1 AIV and the herpes virus of turkeys (HVT) as the vector, Re-6 = Inactivated H5N1 vaccine.*

CV = Co-efficient of variation, NP = number of birds having protective ELISA antobody titre (> 668).

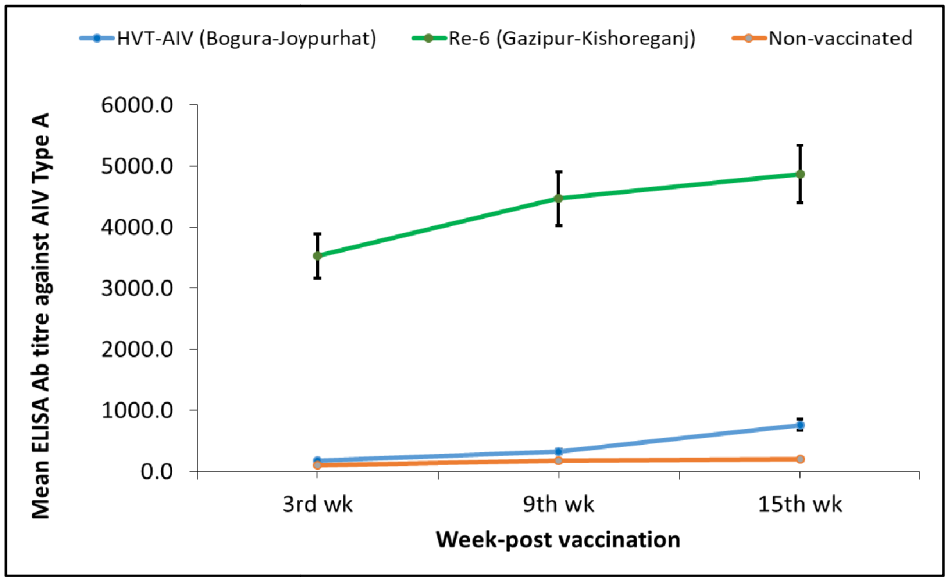

Figure 9. ELISA antibody titre (mean ± standard error) against AI type A in layer birds of different vaccinated and unvaccinated farms at different occasions

Table 4. Biosecurity status of the farms of different districts

Districts	Biosecurity score		
	Good	Moderate	Poor
Bogura		5	1
Joypurhat		4	2
Kishoregonj		5	1
Gazipur	6		

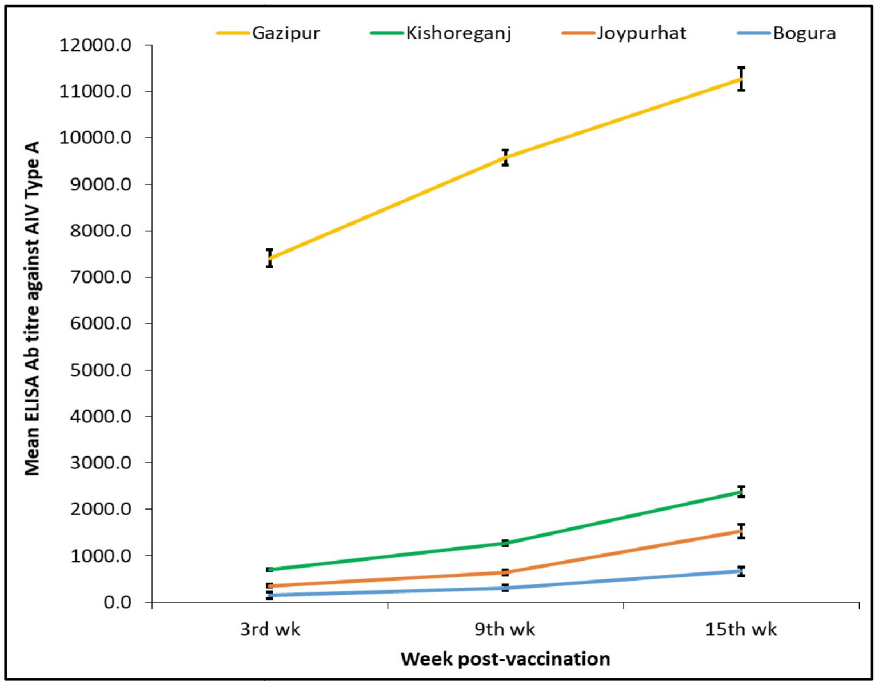

Figure 10. District-wise ELISA antibody titre (mean ± standard error) against AI type A in layer birds of different vaccinated farms at different occasions

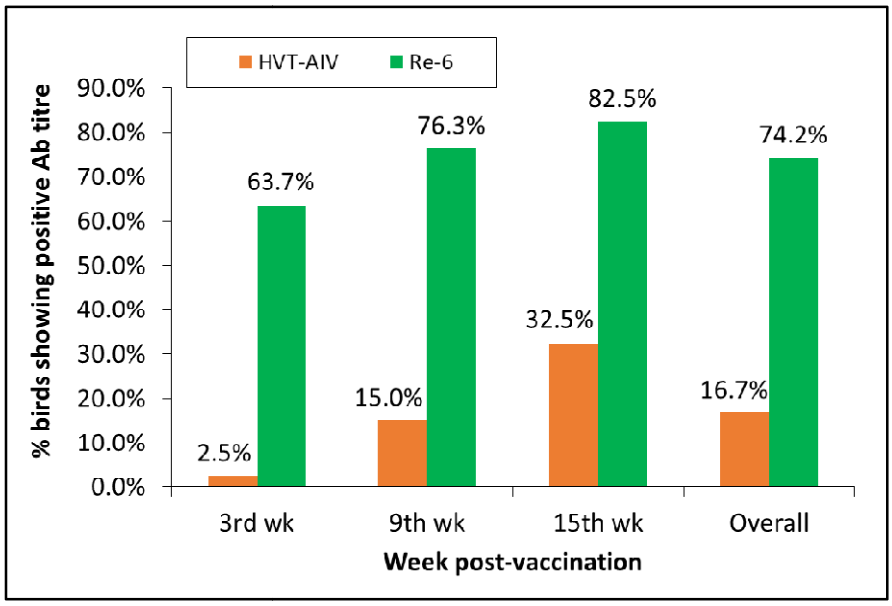

Figure 11. Percent (%) birds showing protective ELISA antibody titre against Avian Influenza type A at different time points in vaccinated layer birds.
HVT-AIV= Recombinant AI vaccine, which contains the hemagglutinin (H) gene of an H5N1 AIV and the herpes virus of turkeys (HVT) as the vector, Re-6 = Inactivated H5N1 vaccine.

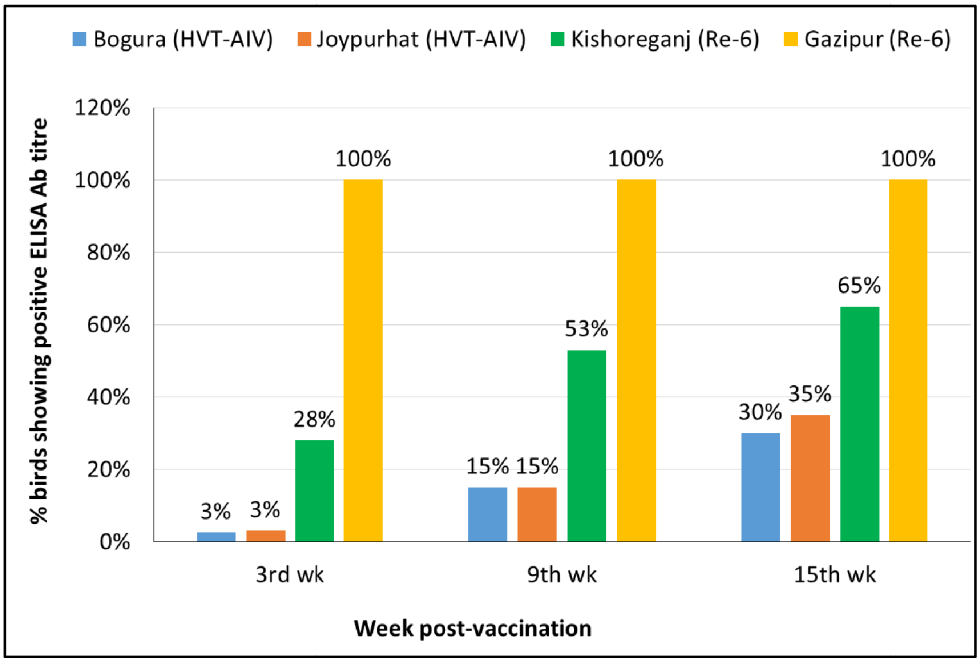

Figure 12. District-wise percent (%) birds showing protective ELISA antibody titre against Avian Influenza type A at different time points in vaccinated layer birds.

Assessment of the immune escaped AI virus (AIV) shedding in AI vaccinated flocks

Positive reactors based on percent inhibition (PI) values for H5 and H9 subtypes

Among the 218 sera samples from vaccinated flock which had protective antibody titre against Avian Influenza Type A only, 118 (54.1%) (Re-6, 86, HVT-AIV, 32) samples found positive in terms of PI values against H5 subtype (Table 5). However 23 among randomly selected 50 birds, which were vaccinated with HVT-AIV but did not have protective ELISA antibody titre against AIV Type A, found with positive PI values against H5 antigen. One sample from unvaccinated bird that was positive for type A, had positive PI value against H5. Interesting to note that 46 (21%) samples were found with positive PI values against H9 antigen that indicate the colonization of H9 subtype.

Table 5. Percent positive birds for H5 subtype in layer birds which had protective ELISA antibody titre against Avian Influenza Type A

Vaccine	Farm ID*	3-week PV		9-week PV		15-week PV		Overall	
		Type A +ve	H5 +ve	Type A +ve	H5 +ve	Type A +ve	H5 +ve	Type A +ve	H5 +ve No. (%)
Re-6	KS1	4	1	7	2	7	2	18	5 (28)
	KS2	5	1	6	2	7	3	18	6 (33)
	KB1			3		6	2	9	2 (22)
	KB2	2		5	1	6	1	13	2 (15)
	GS1	10	5	10	6	10	7	30	18 (60)
	GS2	10	4	10	6	10	6	30	16 (53)
	GSr1	10	5	10	7	10	6	30	18 (60)
	GSr2	10	6	10	6	10	7	30	19 (63)
Total (Re-6)		51		61		66		178	86 (48)
HVT-AIV	BS1			2	1	3	2	5	3 (60)
	BS2					3	2	3	2 (67)
	BSp1			2	1	3	3	5	4 (80)
	BSp2	1	1	2	2	3	3	6	6 (100)
	JS1			2	1	2	2	4	3 (75)
	JS2					3	3	3	3 (100)
	JK1			2	1	4	3	6	4 (67)
	JK2	1	1	2	2	5	4	8	7 (88)
Total (HVT-AIV)		2	2	12	8	26	22	40	32 (80)

**Farm ID used in order to maintain farms anonymity, derived from abbreviation for the district and upazila in which the farm was located.Positive PI value ≥ 50, PV = Post vaccination.*

Optical dencity (OD) values for different vaccines against N1 antigen

None of the flock was immune against N1 antigen as evidenced by the values of mean OD. However, only two individual samples collecetd from Re-6 vaccinated birds of Kishoreganj sadar and Gazipur sadar upazilas were found with positive OD values at 9 week post vaccination (Table 6).

Table 6. Optical density (OD) values with number of positive birds for N1 subtype, which have shown protective ELISA antibody titre against Avian Influenza Type A

District	Upazila	Farm ID	Vaccine	No. of AIV-A positive (n = 219)	OD values for N1 Mean ±SD (No. of N1 positive birds)			Judgement
					3 week PV	9 week PV	15 week PV	
Bogura	Sadar	BS1	HVT-AIV	0+2+3	-	.070±.001(0)	.094±.058 (0)	Negative
		BS2		3	-	-	.124±.032 (0)	Negative
		BS3	NV	0	-	-	-	Negative
	Shahjahanpur	BSp1	HVT-AIV	0+2+3	-	.088±.045(0)	.125±.006 (0)	Negative
		BSp2		1+2+3	.096 (0)	.099±.081(0)	.103±.083 (0)	Negative
		BSp3	NV	0	-	-	-	Negative
Joypurhat	Sadar	JS1	HVT-AIV	0+2+2	-	.068±.003(0)	.083±.018 (0)	Negative
		JS2		0+0+3	-	-	.091±.012 (0)	Negative
		JS3	NV	0	-	-	-	Negative
	Kalai	JK1	HVT-AIV	0+2+4	-	.078±.016(0)	.084±.018 (0)	Negative
		JK2		1+2+5	.070 (0)	.081±.002(0)	.092±.007 (0)	Negative
		JK3	NV	0	-	-	-	Negative
Kishoreganj	Sadar	KS1	Re-6	4+7+7	.085±.061 (0)	.097±.064(1)	.073±.013 (0)	Negative
		KS2		5+6+7	.076±.030 (0)	.081±.007(0)	.074±.005 (0)	Negative
		KS3	NV	1	-	-	0.077 (0)	Negative
	Bazitpur	KB1	Re-6	0+3+6	-	.080±.012(0)	.090±.008 (0)	Negative
		KB2		2+5+6	.068±.007 (0)	.070±.028(0)	.086±.015 (0)	Negative
		KB3	NV	0	-	-	-	Negative
Gazipur	Sadar	GS1	Re-6	10+10+10	.062±.038 (0)	.098±.051(1)	.102±.062 (0)	Negative
		GS2		10+10+10	.067±.051(0)	.072±.051(0)	.091±.057(0)	Negative
		GS3	NV	0	-	-	-	Negative
	Sreepur	GSr1	Re-6	10+10+10	.069±.042(0)	.102±.071(0)	.113±.059 (0)	Negative
		GSr2		10+10+10	.095±.059 (0)	.110±.051(0)	.122±.021 (0)	Negative
		GSr3	NV	0	-	-	-	Negative

**Farm ID used in order to maintain farms anonymity, derived from abbreviation for the district and Upazila in which the farm was located. NV = Non-vaccinated*
Critical Value (CV) = 0.217
Positive Judgement = Mean OD > CV, Negative Judgement = Mean OD < CV.

Results of qPCR

Among the 98 cloacal swab samples from vaccinated flocks, 16 (16.3%) samples were positive for AIV type A (M gene), of which, 9 were positive for AIV subtype H5N1, and the remaining 7 were positive for AIV subtype intermediate (Table 7). Moreover,two samples from unvaccinated birds were positive for H5N1.

Table 7. Results of qPCR of cloacal swab samples collected from AI vaccinated flocks

AIV type and subtype	Positivity (n= 98)
Influenza type A	16 (16.3%)
Avian influenza subtype H5N1	9 (9.2%)
Avian influenza subtype indeterminate	7 (7.1%)

n = Number of cloacal swab samples

11.2 Discussions

The commercial poultry sector in Bangladesh faced massive attack of avian influenza in 2007 and onwards, and initially *Stamping-out* of the infected flock was applied to combat the situations. But due to failure of the control strategy Highly Pathogenic Avian Influenza (HPAI) became endemic in Bangladesh and the government took up vaccination policy against avian influenza.

Two different commercial vaccine preparations, HVT-AIV and Re-6 were used in the layer farms in our study area. The HVT-AIV vaccine did not produce consistent and protective ELISA antibody titre against Avian Influenza type A. Among many factors of poor immune response, poor biosecurity practice is one of them. The biosecurity score of the HVT-AIV vaccinated farms was moderate to poor. The Re-6 vaccine was found to develop consistent and protective ELISA antibody titre against Avian Influenza type A in the flocks with good biosecurity. However, in flocks with moderate to poor biosecurity the titre was not consistent. The findings agree with the reports of Tarigan et al. (2018) who evaluated seven commercial AI vaccines and found that none of the vaccines were consistently effective. Irrespective of vaccine type 218 (45.4%) birds had protective ELISA antibody titre against Avian Influenza A.

Irrespective of area and farms the Re-6 vaccine induced significantly higher mean ELISA antibody titre in birds than the HVT-AIV vaccine. HVT-AIV vaccine is a recombinant AI vaccine, which contains only the hemagglutinin (H) gene of an H5N1 avian influenza virus and the herpes virus of turkeys (HVT) as the vector but missing the nuraminidase (N1) gene. On the other hand Re-6 is an inactivated H5N1 vaccine which contains both the hemagglutinin (H) and nuraminidase (N1) gene. These findings agree with the statement of Kapczynski et al. (2017) who observed inactivated vaccines provided superior protection against the H5N8 and H5N2 viruses in a single vaccination strategy compared with the recombinant vaccines.

Significant variation in mean protective ELISA antibody titre was observed at different occasions of post vaccination in case of Re-6 vaccine. The age at primary vaccinations was different for all the farms vaccinated with Re-6 vaccine. Better immune response was observed in birds which were vaccinated between 5 to 9 weeks of age than the birds vaccinated on age above the mentioned time period. Young birds possess more active immune response to vaccination (Lavoie et al., 2007; Davison et al., 2008).

The level of flock immunity was good in some flocks those had good biosecurity and poor in some other vaccinated flocks though the antibody titre of some individual birds reached above the protective ELISA antibody titre (>668). The poor response might be due to single dose vaccination strategy of the farms along with poor biosecurity practices. A booster dose of vaccine after the primary vaccination provides superior immune response than single dose vaccination protocol (Bertran et al., 2015). All the farms under this study followed single dose vaccination protocol for both type of vaccine though the manufacturer's instruction for Re-6 vaccine was to go for a booster vaccination at 5 week post primary vaccination. In case of endemic countries no single dose vaccination regime for High Pathogenic Avian Influenza is recommended (van den Berg et al., 2008).

Poor immune response was observed in birds vaccinated with HVT-AIV at three week-post vaccination which might be due to the presence of maternal derived antibody. HVT-AIV vaccine is administered at day old chick. Maternal derived antibody interferes with the avian influenza vaccine up to 20 days of age (De Vriese et al., 2010). Better immune response was observed in birds at 9 week post vaccination than 3 week and 15 week post vaccination samples.

Though 218 sera samples had protective antibody titre against Avian influenza Type A only 118 (54.1%) of them showed positive percent inhibition value against H5 subtype. The protective ELISA antibody titre against Avian Influenza Type A in other 100 (45.9%) samples might be due to colonization of other subtype of low pathogenic Avian Influenza virus.

Interesting to note that Type A positive 218 sera samples were tested for H9 subtype, and 46 (21%) samples showed positivity against H9 subtype though neither of the vaccines contained H9 antigen. So it is clear that the protective ELISA antibody titre against Avian Influenza Type A was not due to vaccine. This might be due to colonization of other H9 subtype. There is evidence of circulating non-pathogenic H9N2 subtype in Bangladesh (Negovetich et al., 2011). We also tested the Type A positive samples for antibody against N1 subtype where only two birds were found positive for Type A virus. The contribution of N1 to immunity is not clear yet and remains to be studied (Pushko et al. 2017).

In the vaccinated birds, around 16% birds were virus shedder at different occasions. It indicates even after vaccination there may be virus shedding in the flocks that may facilitate further spread of the virus. It warrants for revisiting of the vaccination strategy.

12. Research highlight/findings:

I. Re-6 vaccine produced protective and consistent humoral Ab titre against AIV type A in farms of Gazipur district those had good biosecurity compared to farms of Kishoreganj district, which had moderate to poor biosecurity.

II. Among 480 birds vaccinated with either of the two vaccines (HVT-AIV and Re-6), 218 (45.4%) birds found to have positive ELISA or protective antibody titre against AIV Type A. The overall percentage of positive birds was higher (74.2%) for Re-6 vaccinated flocks than HVT-AIV vaccinated flocks (16.7%)

III. The percentage of positive reactors increased over time at 3-, 9-, and 15-week post vaccination. Among the Re-6 vaccinated birds, 63.7%, 76.3% and 82.5% birds found to have positive ELISA antibody titre at 3-, 9-, and 15-week post vaccination, respectively, while 2.5%, 15% and 32.5% were positive among HVT-AIV vaccinated birds at the same time point.

IV. HVT-AIV vaccine failed to produce protective humoral Ab titre against AIV type A.

V. Positive H9 titre was found in 21% vaccinated birds though H9 vaccine was not practiced in the studied flocks.

VI. AI vaccinated birds (16%) shed virus in the droppings that may facilitate further spread of the virus.

B. Implementation Position

1. Procurement:

Description of equipment and capital items	PP Target		Achievement		Remarks
	Phy (#)	Fin (Tk)	Phy (#)	Fin (Tk)	
(a) Office equipment					
(b) Lab &field equipment	03	3,20,000	03	3,19,700	
(c) Other capital items					

2. Establishment/renovation facilities: Not applicable

Description of facilities	Newly established		Upgraded/refurbished		Remarks
	PP Target	Achievement	PP Target	Achievement	

3. Training/study tour/ seminar/workshop/conference organized: Not applicable

Description	Number of participant			Duration (Days/weeks/ months)	Remarks
	Male	Female	Total		
(a) Training					
(b) Workshop					

C. Financial and physical progress

Fig in Tk

Items of expenditure/activities	Total approved budget	Fund received	Actual expenditure	Balance/ unspent	Physical progress (%)	Reasons for deviation
A. Contractual staff salary	808875	735133	688535	47673	93.66	
B. Field research/lab expenses and supplies	1116500	1116500	1115160	1340	99.88	
C. Operating expenses	214000	214000	214000	0.00	100	
D. Vehicle hire and fuel, oil & maintenance	30000	30000	30000	0	100	
E. Training/workshop/seminar etc.	0	0	0	0		
F. Publications and printing	75000	43073	15000	28073	34.82	
G. Miscellaneous	50000	50000	50000	0	100	
H. Capital expenses	320000	320000	319700	300	99.91	
Total	**2614375**	**2508706**	**2432395**	**76311***	**96.96**	

**Refund to NATP- Tk. 76311*

D. Achievement of Sub-project by objectives: (Tangible form)

Specific objectives of the sub-project	Major technical activities performed in respect of the set objectives	Output (i.e. product obtained, visible, measurable)	Outcome (short term effect of the research)
To measure the antibody titre against AIV Type A in commercial layer birds	Sampling of blood; Indirect ELISA	- Among 480 birds vaccinated with either of the two vaccines (HVT-AIV and Re-6), 218 (45.4%) birds found to have positive ELISA or protective antibody titre against AIV Type A. - The overall percentage of positive birds was higher (74.2%) for Re-6 vaccinated flocks than HVT-AIV vaccinated flocks (16.7%) - The percentage of positive reactors increased over time at 3-, 9-, and 15-week post vaccination. Among the Re-6 vaccinated birds, 63.7%, 76.3% and 82.5% birds found to have positive ELISA antibody titre at 3-, 9-, and 15-week post vaccination, respectively, while 2.5%, 15% and 32.5% were positive among HVT-AIV vaccinated birds at the same time point. - Re-6 vaccine produced protective and consistent humoral Ab titre against AIV type A in farms from Gazipur district those had good biosecurity compared to farms of Kishoreganj district, which had moderate to poor biosecurity. - HVT-AIV vaccine failed to produce protective humoral Ab titre against AIV type A.	Farmers will be aware in choosing right vaccine to protect their birds against AIV infection
To determine subtype specific immune response through ELISA	Sampling of blood; Competitive ELISA	- Percent Inhibition values were known for H5 and H9 following vaccination and out of the 218 sera samples with the protective antibody titre against Avian influenza Type A only 118 (54.1%) showed positive percent inhibition value against H5 subtype. - Positive H9 titre was found in 21% vaccinated birds though H9 vaccine was not practiced in the studied flocks. - None of the flock was immune against N1 antigen as evidenced by the values of mean OD.	
To assess the immune escaped AI virus (AIV) shedding in AI	Sampling of cloacal swabs; Real-time PCR	- Around 16% of the AI vaccinated birds were found to shed virus in the droppings that may facilitate further spread of the virus.	The knowledge will help in AI vaccination

vaccinated flocks			policy support

E. Materials Development/Publication made under the Sub-project:

Publication	Number of publication		Remarks (e.g. paper title, name of journal, conference name, etc.)
	Under preparation	Completed and published	
Technology bulletin/ booklet/leaflet/flyer etc.	-	-	-
Journal publication	1		*Title*: Assessment of immune responses to avian influenza vaccination in commercial layer birds in Bangladesh *Journal name*: Vaccine
Information development	-	-	-
Other publications (MS Thesis)		1	*Thesis Title*: Assessment of immune responses to avian influenza vaccination in commercial layer birds in Bangladesh *MS Fellow*: Md. Shafiul Islam *Submitted to the Department of Medicine, BAU, Mymensingh in June 2018*

F. Technology/Knowledge generation/Policy Support (as applied):

i. Generation of technology (Commodity & Non-commodity)

None

ii. **Generation of new knowledge that help in developing more technology in future**

AI vaccinated birds (16%) shed virus in the droppings that may facilitate further spread of the virus; Positive H9 titre was found in 21% vaccinated birds though H9 vaccine was not practiced in the studied flock

iii. Technology transferred that help increased agricultural productivity and farmers' income

None

iv. Policy Support

- AI vaccination should be done in flocks where good biosecurity is ensured;
- The vaccination strategy is to be revisited as 16% of AI vaccinated birds shed virus in the droppings;
- Vaccination of birds against H9 is to be considered as 21% birds showed positive H9 antibody titre

G. Information regarding Desk and Field Monitoring

i) **Desk Monitoring (description & output of consultation meeting, monitoring workshops/seminars etc.):**

ii) **Field Monitoring (time & No. of visit, Team visit and output):**

H. Lesson Learned (if any)

Nothing particular

I. Challenges (if any)

None

Signature of the Principal Investigator
Date ……
Seal

Counter signature of the Head of the organization/authorized representative
Date ……..
Seal

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