Project ID: 556

Competitive Research Grant

Sub-Project Completion Report

on

Development and evaluation of formalin killed inactivated egg drop syndrome virus vaccine using local isolates

Project Duration

May 2017 to September 2018

Department Of Microbiology and Hygiene
Bangladesh Agricultural University
Mymensingh-2202

Submitted to

Project Implementation Unit-BARC, NATP 2
Bangladesh Agricultural Research Council
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Project Implementation Unit
National Agricultural Technology Program-Phase II Project (NATP-2)
Bangladesh Agricultural Research Council (BARC)
New Airport Road, Farmgate, Dhaka – 1215
Bangladesh

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The execution of CRG sub-project has successfully been completed by Department of Microbiology and Hygiene, Bangladesh Agricultural University using the research grant of USAID Trust Fund and GoB through Ministry of Agriculture. We would like to thanks to the World Bank for arranging the grant fund and supervising the CRGs by BARC. It is worthwhile to mention the cooperation and quick responses of PIU-BARC, NATP 2, in respect of field implementation of the sub-project in multiple sites. Preparing the project completion report required to contact a number of persons for collection of information and processing of research data. Without the help of those persons, the preparation of this document could not be made possible. All of them, who made it possible, deserve thanks. Our thanks are due to the Director PIU-BARC, NATP 2 and his team who have given their whole hearted support to prepare this document. We hope this publication would be helpful to the agricultural scientists of the country for designing their future research projects in order to technology generation as well as increasing production and productivity for sustainable food and nutrition security in Bangladesh. It would also assist the policy makers of the agricultural sub-sectors for setting their future research directions.

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Acronyms

EDS	: Egg Drop Syndrome
HA	: Hemagglutination Activity
EID_{50}	: Embryo Infective Dose 50
HI	: Hemagglutination Inhibition
ELISA	: Enzyme Linked Immunosorbant Assay
EDE	: Embryonated Duck Egg
EDSV	: Egg Drop Syndrome Vaccine
FAO	: Food and Agricultural Organization
AAF	: Amniotic Allantoic Fluid
cRBC	: Chicken Red Blood Cell
PBS	: Phosphate Buffer Saline
ND	: Newcastle Disease
IB	: Infectious Bronchitis
BAURES	: Bangladesh Agricultural University Research System
BARC	: Bangladesh Agricultural Research Council

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Executive Summary

Egg drop syndrome is one of the most common barriers in poultry production especially in layer birds that is characterized by sudden drop in egg production and production of shell less or soft shelled eggs. To protect the layer birds from this disease inactivated oil based vaccine was prepared using local isolates of EDS-76 virus obtained from a previously implemented project of BARC under NATP: Phase- 1. Initially, for reactivation, the virus EDS-76 was propagated into 11 days old duck embryo and allantoic fluid was harvested. The HA titre and EID_{50} of the virus suspensions after reactivation were $log_2 14$ and $log10^{10.6}$ /ml. For vaccine preparation, the virus was inactivated by 0.12% formalin at a concentration of EID_{50} $log10^{8.6}$ /ml. Then after performing safety and sterility tests the inactivated virus suspension was adjuvanated either with montanide and was designated as BAU-EDS1) or with a mixture of oil base containing arlacil, tween-80 and liquid paraffin and was designated as BAU-EDS2. A total of 80 layer birds (16 weeks old) were divided into four groups of A, B, C and D. The birds of group A, B and C were vaccinated with experimental BAU-EDS1, BAU-EDS2 and commercial EDS (Nobilis®) vaccines respectively. Birds of group D was kept as unvaccinated control. The sera samples were collected at 16, 17, 18, 19, 20, 22, 24, 28, 32, 34 and 35 weeks of age and subjected to HI and ELISA tests. Before vaccination the serum titres of the birds determined by HI and ELISA were below protective level. At 17 weeks the HI titres were 80, 96, 120, 00 and the ELISA titre (S/P ratio) were 0.421679, 0.527013, 0.574927 and 0.011983 in groups of A, B, C and D respectively. Gradually the antibody titres of the vaccinated birds were increased and reached highest at 20 weeks of age with the HI titres of 448, 576 and 448 in groups of A, B and C respectively. After challenge with virulent EDS-76 virus at 32 weeks, there was decreased egg production (47.14%) with miss-shaped, soft-shelled and shell less eggs in unvaccinated birds (group D). On the contrary, there was no drop in egg production and no change of the quality of eggs in the birds vaccinated with experimental vaccines (groups A and B) and commercial vaccine (group C). However, in the vaccinated groups of birds there was little decrease in egg production but no production of shell less or soft shelled eggs. The results of the present study revealed that the experimental vaccines BAU-EDS1 and BAU-EDS2 are able to induce high antibody titres and protect the layer birds against virulent EDS virus as like as commercial vaccine. Of the two different experimental vaccines BAU-EDS2 induced higher HI antibody titre (576) compared to BAU-EDS1 (448) and the commercial vaccine (448) Nobilis®. It is therefore recommended that the vaccine could be used to protect layer birds from EDS-76. However, farm level study is needed involving bigger flocks for validation of the vaccine before declaring the vaccine effective and safe to use for protecting against EDS-76 virus infection in the commercial layer farms of Bangladesh.

CRG Sub-Project Completion Report (PCR)

A. Sub-project Description

1. **Title of the CRG sub-project:**
   Development and evaluation of formalin killed inactivated egg drop syndrome virus vaccine using local isolates

2. **Implementing organization:**
   Department of Microbiology and Hygiene**,** Faculty of Veterinary Science
   Bangladesh Agricultural University

3. **Name and full address with phone, cell and E-mail of PI/Co-PI (s):**

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4. **Sub-project budget (Tk):**
   4.1 Total: 16,23,365 BDT
   4.2 Revised (if any): Not applicable

5. **Duration of the sub-project:**
   5.1 Start date (based on LoA signed): 16 May 2017
   5.2 End date : 30 September 2018

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

Poultry is a rapidly growing sub-sector in Bangladesh. This sub-sector is particularly important in the sense that it is a significant source of supply of protein and nutrition in a household's nutritional intake. Every year several disease outbreaks occur in poultry farm throughout the country resulting huge economic losses due to high morbidity and mortality. Among them Egg drop syndrome (EDS) is the world's most feared disease in layer chickens. This disease is characterized

by sudden drops in egg production with poor shell quality eggs due to EDS-76 virus infection. The drop in egg production in layer birds has become a major concern in Bangladesh due to the enormous economic burden faced by the farmers. The loss incurred by poultry industry due to reduced productivity, culling and cost of medicine is considered to be often greater than loss due to mortality.

Jahangir *et al.* (2009) first reported the presence of EDS virus (EDSV) in Bangladesh. Since then there was no research or study on EDSV in Bangladesh. However, recently we have isolated EDS-76 virus from Bangladesh which were characterized molecularly and biologically. The total research work was carried out under the project entitled "Isolation and molecular characterization of egg drop syndrome (EDS-76) virus in Bangladesh" from June, 2012 to July 2014 funded by "SPGR sub-projects, PIU-BARC, NATP: Phase-1". Under this project 3 copies of the virus were isolated from different parts of the country. Later formalin killed EDSV vaccine was developed with the local isolates and tested in small scale on layer chicken flock to observe its efficacy. It was therefore hoped that if a vaccine against EDS-76 is developed, using those local isolates from the previous research project, will produce higher antibody titer including 100% protection like as commercial EDSV vaccine. The present research project was thus undertaken to develop an inactivated vaccine against EDS using local isolate of Egg drop syndrome virus and testing its efficacy in commercial layer birds.

7. Sub-project goal:

The goal of the present research project was to develop formalin killed EDSV vaccine to prevent EDS-76 disease for increasing egg production with the ultimate goal of farmers benefit.

8. Sub-project objective (s):

- Development of formalin killed oil adjuvanated EDSV vaccine.
- Determination of protective efficacy of experimental vaccine against EDS-76 virus in layer birds under experimental conditions.
- Application of vaccine at commercial layer farms.

9. Implementing location (s):

Department of Microbiology and Hygiene, Bangladesh Agricultural University

10. Methodology in brief:

10.1. Development of formalin killed oil adjuvanated EDSV vaccine

Preparation of seed virus for the development of EDSV vaccine:

a) Collection of duck eggs

Duck eggs with the history of high fertility were obtained from duck farms, Mymensingh. The eggs were washed and disinfected with 70% alcohol. The eggs were incubated at 37˚ C maintaining relative humidity (~85%) for 10-11 days. The eggs were turned twice to get the best possible healthy

embryos for the experiment. The well developed and healthy embryos were used for propagation of EDS-76 virus and determination of $EID_{50.}$ The temperature, humidity and turning was ensured through regular inspection; twice daily. At day 11 the embryonated duck eggs (EDE) were ready for virus inoculation.

b) Reactivation of virus into EDE and determination of virus titre:

The locally isolated and characterized egg drop syndrome virus (EDSV) was used for the preparation of vaccine. For this purpose 0.2 ml of virus suspension was inoculated into 11 days old duck embryos through allantoic cavity route followed by incubation at 37^0 C for 5 days. After 5 days of incubation the embryos were chilled at 4^0 C. The allantoic fluid was harvested and checked for hemagglutination activity by hemagglutination assay (HA) test.

EID_{50} determination was done according to standard procedures described by Reed and Muench (1938) and using EID_{50} calculators, as described by FAO (2002). For this purpose, 100 embryonated duck eggs were used. After 5 days of incubation, the allanto-amniotic fluid (AAF) was harvested and pooled together. The pooled allantoic fluid containing EDS viruses was clarified by centrifugation at 5000 rpm for 10 minutes. The hemagglutination activity of pooled AAF and embryo infective dose 50 (EID_{50}) by Reed & Muench method was determined using embryonated duck eggs.

At first a serial ten-fold dilutions of the virus suspension (HA titre = 14log2) was prepared in a series of eppendorf tubes. As the HA titre of the virus was high, a range of dilution from 10^{-1} to 10^{-14} were made. The initial few dilutions were not inoculated. A 0.1ml suspension from each dilution starting from 10^{-5} to 10^{-14} was inoculated into 5 well developed healthy embryos, through allantoic cavity route. The embryos were then incubated for 5 days. Any embryo died within 24 hours was discarded. All the embryos were chilled after 5 days of incubation and AAF were collected in separate vials. Hemagglutination activity of each AAF was examined by micro-titre HA test and recorded the result. The EID_{50} was calculated by Reed & Muench method to establish the dilution by calculating the (Reed and Muench, 1938) index. The index was then added to the dilution that produced the percentage of EDE infected immediately above 50 percent, i.e. 10^{-8}.

The Reed and Muench formula to calculate the index is:

$$\text{Index} = \frac{(\%\text{ infected at dilution immediately above 50\%}) - 50\%}{(\%\text{ infected at dilution immediately above 50\%}) - (\%\text{ infected at dilution immediately below 50\%})}$$

c) Inactivation of virus with formalin:

The AAF was transferred to a sterilized glass bottle and formalin was added to virus suspension at the rate of 0.12%. The mixture was properly mixed by continuous stirring using magnetic stirrer and incubated at 37^0 C for 48 hours. After mixing the mixture was kept at refrigerator for proper inactivation of virus. The complete inactivation was confirmed by inoculation of the formalin killed virus into EDS free embryonated duck eggs for at least two consecutive times and observing for no growth of the virus.

d) Sterility Test:

The inactivated virus suspension was tested for sterility to determine any bacterial or fungal growth in inactivated vaccine. For this reason 10 ml of AAF was centrifuged at 600xg for 15 minutes. The sediment was streaked onto Nutrient agar, Blood agar, MacConkey's agar and Sabouraud Dextrose agar. The agar plates were incubated at 37^0 C for 24 to 48 hours and observed for microbial growth. The absence or presence of microbial growth in the media indicated the sterility of the experimentally prepared vaccine.

e) Safety test:

A 0.2 ml of inactivated AAF was inoculated into 11 days old embryonated duck eggs via allantoic cavity route. After 5 days of incubation, the embryos were chilled in refrigerator. The AAF was harvested and checked for its hemagglutination activity. No hemagglutination or little HA activity indicated that the inactivated viral suspension is safe for vaccine preparation.

f) Preparation of oil-based EDSV vaccine:

The virus suspension in AF, having HA titre $\log 2^{14}$ was used for oil based vaccine preparation. EDSV vaccine was prepared by mixing of Montanide ISA 71 R VG (Seppic) with the inactivated virus suspension and was named as BAU-EDS1. Another vaccine was prepared by mixing one part of virus suspension with four parts of oil base which was named as BAU-EDS2. The oil base consists of oil phase emulsifier Arlacil (Span 80), Tween-80 and white paraffin oil. Oil-based EDS-76 vaccine was prepared as described by (Yaqub *et al.,* 1998). At first white paraffin oil and arlacil were added in a beaker. Then the mixture was mixed in magnetic stirrer until forming a homogenous foamy mixture. Finally the tween-80 and the virus suspension were slowly added to the mixture and stirred for 2 hours to make a uniform suspension. The adjuvanted vaccines characterized by creamy white color were packed in plastic bottle and stored at 4˚ C until use. The viscosity and stability of the experimental vaccines were checked regularly by storing at 4^0 C for 6 months.

10.2. Determination of protective efficacy of experimental vaccine against EDS-76 virus in layer birds

a) Vaccination of birds:

A total of 80 layer birds aged 16 weeks were divided into four groups consisting of A, B, C and D. The birds of group A, B, C were vaccinated with BAU-EDS1, BAU-EDS2, commercial EDS vaccine Nobilis® respectively and the birds of group D were kept as unvaccinated control. Sera samples were randomly collected from birds of each group at day 0, 7, 14, 21, 28, 45, 60, 90, 120, 134 and 141. The antibody titres against EDS vaccines were determined by HI and the ELISA tests.

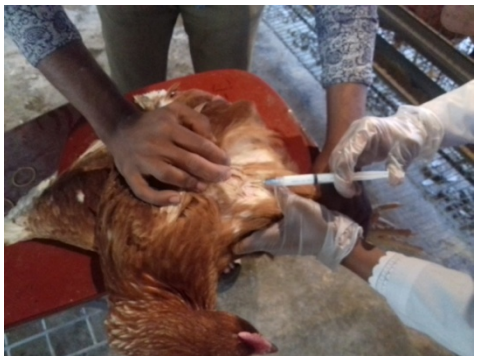


Figure 1. Vaccination of birds

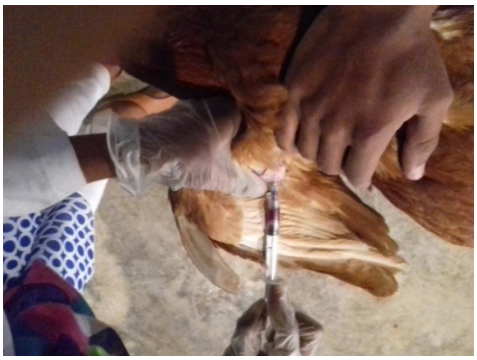


Figure 2. Collection of blood samples from vaccinated birds

b) Challenge of layer birds:

The Birds from all the vaccinated and unvaccinated groups were challenged with virulent EDS virus @ EID_{50} $10^{10.67}$ /ml. After challenge sera samples were collected and the egg production data of the birds were recorded before and after challenge with virulent EDS virus in all groups of birds.

c) Preparation and preservation of serum:

About 3 ml of blood samples were collected aseptically without anticoagulant from the wing vein of the birds using 5 ml disposable syringe. The blood samples were allowed to clot in the syringe at room temperature and then separated from the wall of the syringe by gentle traction or placed in the incubator at 37˚ C for one hour for retraction of the clot. The syringes were then placed in the refrigerator for about 4 hours. The sera were collected in the sterile centrifuge tube and centrifuged at 1500 rpm for 15 minutes to get more clear serum and stored at -20˚ C until used.

d) Slide HA test for detection of EDS-76 virus:

To perform slide HA test, one or two drops of collected allantoic fluid was taken on a clean glass slide and one or two drops of 2% chicken red blood cell (cRBC) was added and mixed thoroughly. The appearance of clumping of the cRBC on the glass slide within 1 to 2 minute was recorded as the presence of hemagglutinating virus in the allantoic fluid.

e) Microtitre plate hemagglutination (HA) test for determination of virus titre:

The HA titre of the isolated hemagglutinating virus were determined by micro-titre plate hemagglutination test. Fifty microliter (50µl) of PBS was first dispensed into each well of a plastic "U" shaped bottom micro-titre plate. Then 50µl of the virus suspension (i. e. infective AF) was poured in the first well and mixed properly by pipetting. Serial two fold dilutions of 50µl volumes of the virus suspensions were made across the plate. Finally 50µl of 1% (v/v) cRBCs was added into each well. The cell virus mixture was mixed well by stirring the plate gently and kept at room temperature for 45 minutes. The HA unit was determined by observing the agglutination of RBC by virus. The highest dilution of the virus at which the cRBCs were found to form a clump and no formation of button at the center of the well was considered as the end point of the HA activity of the virus sample or 1 HA unit. Finally the titration of the virus of each sample was determination and calculated.

f) Micro-titre plate Hemagglutination Inhibition (HI) test:

The HI test was carried out for the determination of antibody titre of sera collected from vaccinated and unvaccinated groups of chickens. An amount of 50µl of PBS was first poured in each well up to 12^{th} vertical row of micro-titre plate. An equal amount of sera sample (50µl) was added in each well of the 1^{st} column. Serial two fold dilutions of serum ranging from 1:2 to 1:4096 were made up to the wells of 12^{th} row from where an excess amount of 50µl of the mixture was poured off. After this a volume of 50µl of prepared antigen (4HA unit) was added in each well up to the 12^{th} row. Wells in 1^{st} row were maintained as control containing equal volume of 50µl of prepared antigen (4HA unit) suspension and PBS. The plate was kept at room temperature for 1 hour. The 50µl of 1% (v/v) cRBCs was added to each well and gently mixed and kept at room temperature for 45 minutes when the control cRBCs settled to a distinct button.

g) Determination of antibody titre by ELISA test:

Preparation of reagent

1. **Substrate Reagent:** To make substrate reagent, 1 tablet was added to 5.5 ml of substrate buffer and allowed for mixing until fully dissolved (approximately 10 minutes). The reagent was prepared on the day of use that was stable for one week when kept in dark at 4˚C.

2. **Wash buffer:** The contents of one wash buffer sachet was added into one liter of distilled or deionized water and mixed well to dissolve fully.

3. All other kit components were kept ready to use but allowed them to come to room temperature (22-27˚C) before use.

Sample preparation

Each test sample was diluted @ 1:500 in sample diluent reagent. A 2 step dilution procedure was followed:

1. 5µl of sample was taken and pipetted into dilution plate recording the position of each sample on a template.
2. 245µl of sample diluent reagent was added to these wells to make a 1:50 dilution.
3. Second dilution was made by adding 90µl of sample diluent provided to the coated plate (Note- diluent was added to the coated plate first in this example).
4. 10 µl of the 1:50 dilution of samples was taken and added directly to the coated plate. That provided a 1:500 sample dilution on the coated plate.

Test procedure

1. A 100µl of negative control was added into wells of A1 and B1 and 100µl of positive control into wells C1 and D1.
2. Each sample was run in a single well and 100µl of diluted 1:500 samples were added into the appropriate wells. The plates were covered with lid and incubated at room temperature (22-27˚C) for 30 minutes.
3. The contents of the well was aspirated and washed 4 times with wash buffer (350µl per well). The plate was inverted and tapped firmly on absorbent paper until no moisture was visible.

4. A 100µl of conjugate reagent was added into the appropriate wells followed by covering of the plates with lid and incubation at room temperature (22-27˚C) for 30 minutes.
5. The contents of the plate was discarded and washed like as step 3.
6. A 100µl of substrate reagent was added into the each well. The plate was covered with lid and incubated at room temperature (22-27˚C) for 15 minutes.
7. Finally 100µl of stop solution was added to the appropriate wells to stop reaction and read the assay within 30 minutes.
8. The absorbance of microtitre plate was recorded by reading at 405 nm.

Result of analysis of ELISA test

For the test result to be valid the mean negative control absorbance should read below 0.3 and the difference between the mean negative control and the mean positive control should be greater than 0.15. Variance in lab temperatures will lead to lower or higher absorbance values. Test sample values related to the control values and the test will still be valid. The EDS positive control has been carefully standardized to represent significant amounts of antibody to EDS in chicken serum. The relative amounts of antibodies in chicken samples can then be calculated by reference to the positive control. This relationship is expressed as S/P ration (Sample to positive ratio).

Interpretation and calculation of results

Sample with an S/P of 0.5 or greater contain anti-EDS antibodies and are considered positive. Mean of test sample-Mean of negative control/Mean of positive control-Mean of negative control=S/P. The following equation relates the S/P of a sample at a 1:500 dilution to an end point titre.

Log_{10} titre=1.14* Log_{10} (S/P) + 3.156
Antilog = Titre

S/P value	**Titre range**	**Antibody status**
0.499 or less	649 or less	No antibody detected
0.500 or greater	650 or greater	Positive

This test is highly specific for antibodies against Egg Drop Syndrome Virus. To avoid false positive reactions that might occur in rare circumstances confirmation with an established reference method is required for a final diagnosis. For this BioChek software program was used with the EDS kit to calculate S/P values, titres and to provide general flock profiling.

10.3. Application of vaccine at commercial layer farms

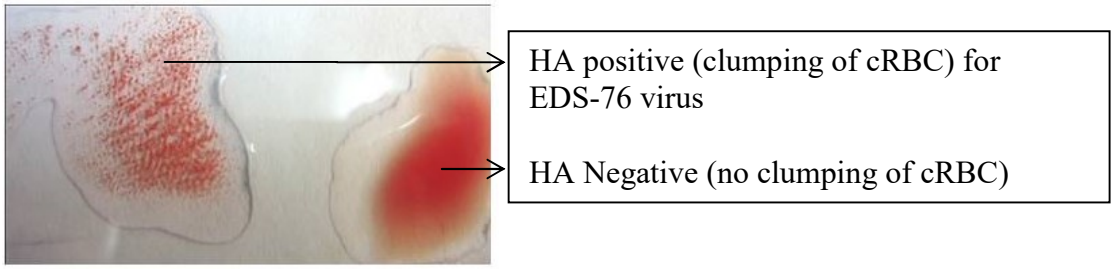
The experiment could not be conducted due to time constraints.

11. Results and Discussion:

11.1: Results

11.1.1. Development of formalin killed oil adjuvanated EDSV vaccine

a) Reactivation of virus: The growth of virus in duck embryo was determined by slide HA test. In slide HA test the EDS virus showed clumping of cRBC (Figure 3). For the preparation of vaccine the titre of the pool virus was determined by HA test (Figure 4) and EID_{50}. In microtitre HA test the HA titre of the pool virus suspension was found to be 14log2 and the EID_{50} was found to be $log_{10}^{10.6}$/ml.



Figur 3: Slide HA test

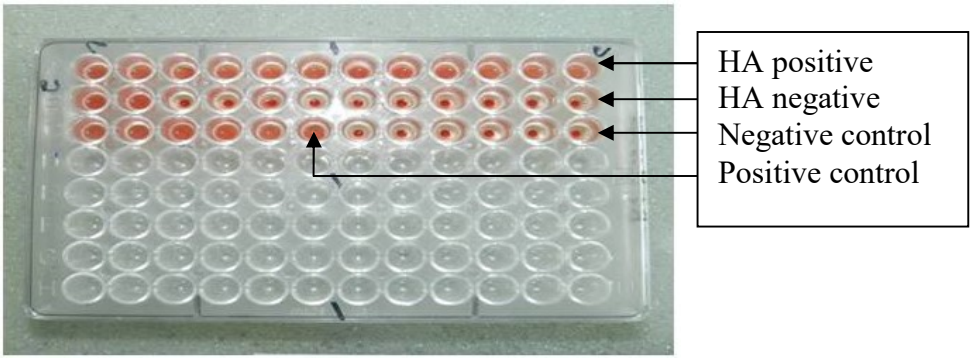


Figure 4: Micro-titre HA test for determination of virus titre

b) Results of inactivation of virus:
In this study EDS-76 virus was diluted with PBS to make a HA titre of 12log2 and EID_{50} of $log_{10}^{8.6}$/ml which was used to make killed vaccine. The virus was inactivated with 0.12% formalin. After inactivation with formalin, the inactivation activity was checked by safety test. To check the contamination with other organism's sterility test was also performed.

c) Sterility test
To check the sterility of the suspension, five blood agar plates were spread with the experimental vaccine followed by incubation of the plates at 37˚ C for over-night. No growth of bacteria on blood agar indicated that the vaccine was sterile (Figure 5).

Figure 5: Sterility test of experimental vaccine
No growth of Bacteria in blood agar media

d) Safety test

For safety test, 0.2ml of inactivated virus was inoculated into 11 days old embryonated duck eggs through allantoic cavity route. After 5 days of incubation, the embryos were chilled in refrigerator. The AF was harvested and checked for hemagglutination activity. No hemagglutination activity was observed which indicate the virus was completely inactivated. Due to complete inactivation, virus did not grow in duck embryo.

e) Adjuvantation of inactivated virus

For adjuvantation of inactivated virus suspension (Figure 6) two kinds of adjuvants were used which were ready to use oil emulsion Montanide and a mixture of paraffin, arlacil, tween-80 and thus two experimental vaccines BAU-EDS1 and BAU-EDS2 were prepared respectively. The adjuvanted vaccines were characterized by creamy-white color (Figure 7) and stored at 4˚C until use. During storage the aqueous part and adjuvanated part of vaccine was separated and remained in upper and lower position respectively.

Figure 6. Oil emulsified adjuvanated experimental EDS vaccines

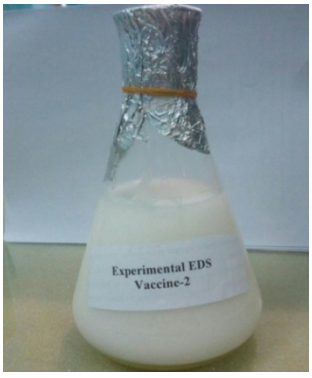


Figure 7. Adjuvanated experimental vaccines- 1 & 2

11.1.2. Determination of protective efficacy of experimental vaccine against EDS-76 virus

a) Antibody titre of vaccinated and unvaccinated birds:
The antibody titres of vaccinated and unvaccinated birds were determined by Micro-titre plate HI and ELISA tests.

Results of HI antibody titre:
The antibody titre against EDS-76 virus of the birds vaccinated with the experimental vaccine is presented in Table 1 and Figure 8. The antibody titre was measured by HI test. Before vaccination the HI antibody titre was found zero at 16 weeks of age. After one week of vaccination at 17 weeks age the antibody titres were found to be 80, 96, 120 and 0 in birds of group A (BAU-EDS1), B (BAU-EDS2), C (Commercial EDS vaccine) and D (Control) respectively. At 18 weeks of age and onward, the mean HI titres of the vaccinated birds of the groups A, B, and C gradually increased up to the age of 20 weeks. At 18 and 19 weeks of age the HI titres were found to be 80, 288, 288 and 288, 576, 384 in groups of A, B and C respectively. At 20 weeks of age (4 weeks of post-vaccination) the antibody titres of all the vaccinated birds reached at peak point such as 448, 576 and 448 in groups of A, B and C respectively. At 22, 24 and 28 weeks of age (6, 8 and 12 weeks of post-vaccination) the antibody titres of all the vaccinated birds started to decline such as 256, 512, 352 and 224, 448, 288 and 160, 352, 256 in groups of A, B and C respectively.

At 32 weeks of age the HI titres were found 160, 288, 224 and 0 in groups of A, B, C and D respectively. The birds of all groups were challenged with the field isolates of EDS-76 virus @ EID_{50} $log_{10}^{10.6}$ per ml at 32 weeks of age. After challenge, at 34 weeks of age the antibody titres were increased to 348, 640, 512 and 156 in groups of A, B, C and D respectively. Finally at 35 weeks of age the HI titres were found to be 352, 512, 352, and 149 in groups of A, B, C and D respectively. The antibody titres in birds of all the vaccinated groups were found higher after 1 week of vaccination (17 weeks) up to the age of 35 weeks compared to unvaccinated control birds.

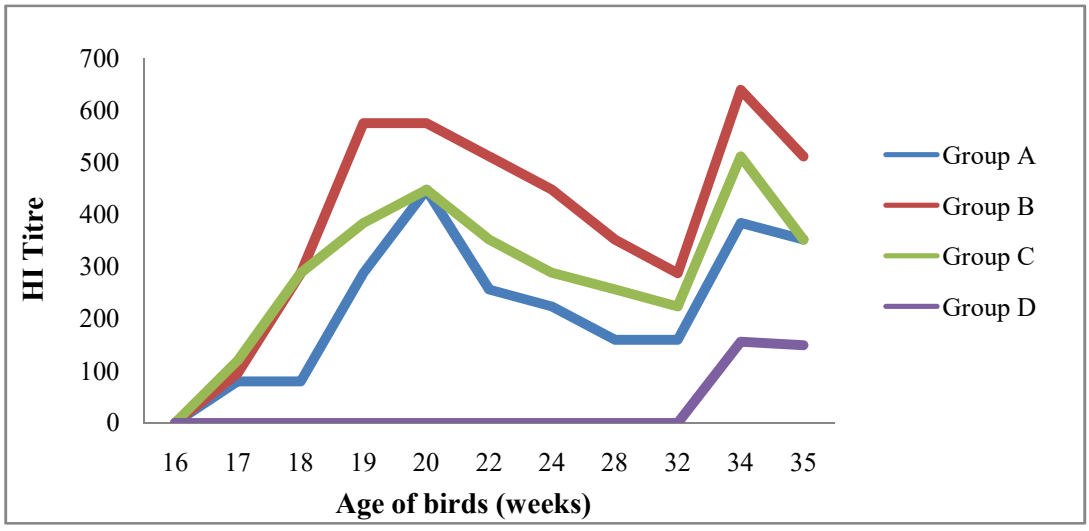


Figure 8. Graphical presentation of HI titres in different vaccinated groups compared with unvaccinated control group

Table 1: Comparative study of HI antibody titres of different vaccinated group of birds

Age of birds (Weeks)	Group A	Group B	Group C	Group D
00	00	00	00	00
17	80	96	120	00
18	80	288	288	00
19	288	576	348	00
20	448	576	448	00
22	256	512	352	00
24	224	448	288	00
28	160	352	256	00
32	160	288	224	00
34	348	640	512	156
35	352	512	352	149

Group A, BAU-EDS1; Group B, BAU-EDS2; Group C, Commercial EDS vaccine Nobilis® and Group D, Unvaccinated control

Results of ELISA test

The ELISA titres against EDS-76 virus of the birds are presented in Table 2 and Figure 10. Micro-titre plate ELISA test was conducted to determine the antibody titres of the birds (Figure 9). The antibody titres started to rise from day 7 and onward which was significant or highly significant compared to the unvaccinated control. After one week of vaccination at 17 weeks of age the ELISA titres were found to be 0.421679, 0.527013, 0.574927 and 0.011983 in the birds of groups A, B, C and D respectively. At 18 weeks of age the ELISA titres of group A, B, C and D gradually increased up to the age of 20 weeks. At 18 and 19 weeks of age the ELISA titres were found 0.530751, 1.224941, 1.307509, 0.082229 and 1.373089, 1.672103, 1.940197, 0.020387 in groups of A, B, C and D respectively. At 20 weeks of age (4 weeks of post-vaccination) the ELISA titres of all age groups of birds reached at peak point such as 2.024465, 2.101257, 2.032966 and 0.028203 in groups of A, B, C and D respectively. At 22, 24 and 28 weeks of age (6, 8 and 12 weeks of post-vaccination) the ELISA titres of all age groups of birds started to decline with the values of 1.086646, 1.996262, 1.996262, 0.117733 and 0.932722, 1.828067, 1.846415, 0.011893 and 0.663948, 1.564399, 1.636425, 0.032622 in groups of A, B, C and D respectively. At 32 weeks of age the ELISA titres were found 0.661233, 1.828067, 1.564399 and 0.016655 in groups of A, B, C and D respectively. The birds of all groups were challenged with the field isolates of EDS-76 virus @ EID_{50} $log_{10}^{8.6}$ per ml at 32 weeks of age. After challenge, at 34 weeks of age the ELISA titres were increased at 2.101937, 2.101257, 2.099898 and 0.477404 in groups of A, B, C and D respectively. Finally at 35 weeks of age the ELISA titres were found 1.996262, 2.032966, 1.940197 and 0.250425 in groups of A, B, C and D respectively.

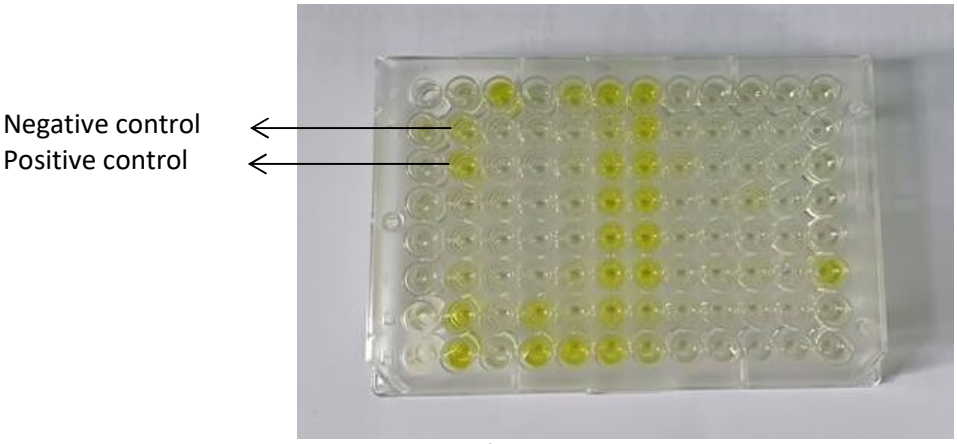


Figure 9. Determination of antibody titres against EDS-76 virus by ELISA test

Determination of protective antibody titres in experimental birds by ELISA

The mean antibody titres of experimental BAU-EDS1, BAU-EDS2 and Nobilis vaccines along with control group at different days of vaccination and also antibody titres of post challenge are given in (Table 2). The mean antibody titres increased gradually after vaccination up to 20 weeks of age. Then the titres were slightly decreased and challenge was given at 32 weeks of vaccination. Then again antibody titres were increased after post challenge. According to kit literature (Biochek, UK), the birds would be protected against EDS-76 virus if the S/P value is 0.500 or greater and antibody titre above 649. The result of this study showed that the antibody titres of the chicken were protective after 20 weeks of vaccination except unvaccinated group. That means vaccinated birds were protected against EDS-76.

Table 2: Comparison of antibody titres (S/P ratio) in different groups of vaccinated and control birds determined by ELISA against EDS-76 virus

Age of birds (Weeks)	Group A	Group B	Group C	Group D
16	0	0	0	0
17	0.421679	0.527013	0.574927	0.011983
18	0.530751	1.224941	1.307509	0.082229
19	1.373089	1.672103	1.940197	0.020387
20	2.024465	2.101257	2.032966	0.028203
22	1.086646	1.996262	1.996262	0.117733
24	0.932722	1.828067	1.846415	0.011893
28	0.663948	1.564399	1.636425	0.032622
32	0.661233	1.828067	1.564399	0.016655
34	2.101937	2.101257	2.099898	0.477404
35	1.996262	2.032966	1.940197	0.250425

Group A, BAU-EDS1; Group B, BAU-EDS2; Group C, Commercial EDS vaccine Nobilis® and Group D, Unvaccinated control.

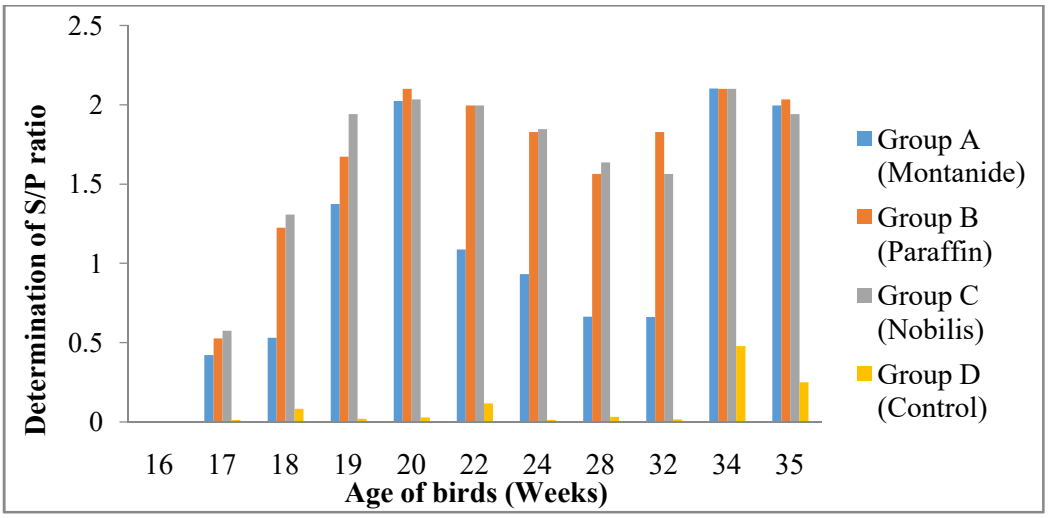


Figure 10. Graphical presentation of S/P ratio in different vaccinated groups compared with unvaccinated control group

b) Results of challenge

After challenge the HI antibody titres of the vaccinated groups were increased from 160 to 348, 288 to 640, 224 to 512 and 00 to 156 in groups of A, B, C and D respectively (Table 1). Egg production of the vaccinated groups remained normal without any change of the quality of eggs (Figure 11) whereas the egg production of control groups drastically declined with soft shelled, shell-less, discolored and miss-shaped eggs (Figure 12). At 4th week of post challenge egg production in unvaccinated group were decreased to 47.14%. The average egg production of the groups A, B, C and D were 85.53%, 91.31%, 92.34% and 64.40% respectively. Egg production in all the four groups of experimental birds, before and after challenge, is presented in Table 3 and Figure 13.

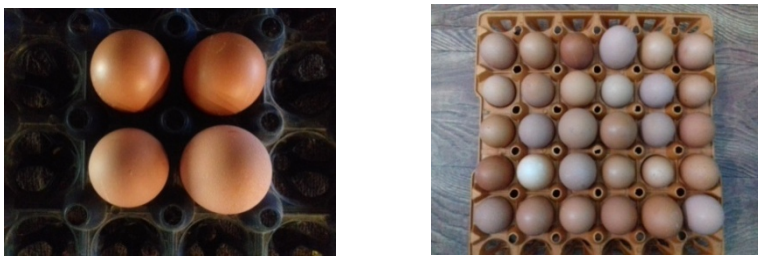


Figure 11. Normal Eggs before challenge with virulent EDS virus

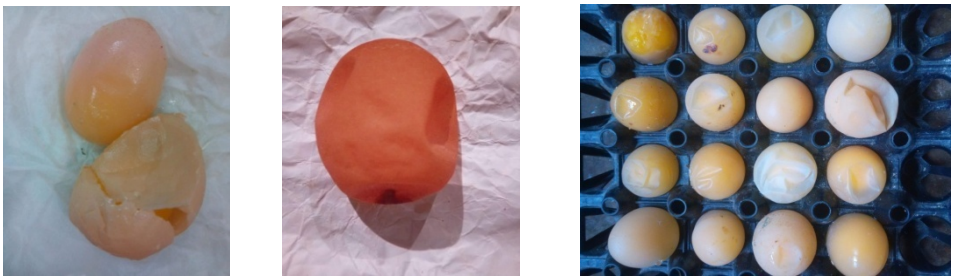


Figure 12. Soft shelled deformed eggs laid by the unvaccinated birds after challenge with virulent EDS virus

Table 3: Egg production status of the experimental birds after challenge with EDS virus

Weeks of post challenge	% of Egg production / week			
	Group A	Group B	Group C	Group D
1st	95.71	96.43	97.14	95.71
2nd	85.29	90	90	68.57
3rd	82.86	89.29	90.71	50.71
4th	82.86	90	90	47.14
5th	84.29	90.71	92.14	58.57
6th	82.14	91.43	94.14	65.71
Average	85.53	91.31	92.34	64.40

Group A, BAU-EDS1; Group B, BAU-EDS2; Group C, Commercial EDS vaccine Nobilis® and Group D, Unvaccinated control.

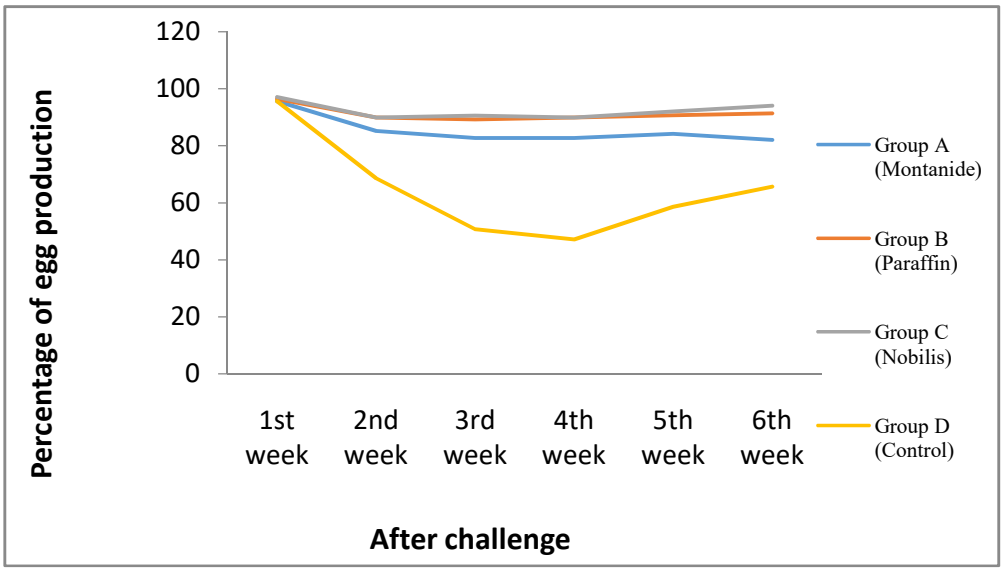


Figure 13. Graphical presentation of egg production in percentage of different vaccinated and unvaccinated group of birds

11.2. Discussion:

The research work was conducted for the development of formalin killed Egg drop syndrome (EDS) virus vaccine from local isolate and to evaluate the efficacy of vaccines in layer birds.

EDS-76 is responsible for sudden decrease in egg production along with production of soft shelled and shell-less eggs (McFerran & Adair, 2003). The production may decrease from 10 to 40% (Yamaguchi *et al.,* 1980 and McFerran, 2003) or more which is closely related in the present study and the course of the disease may extend from few days to few weeks, causing severe economic loss to the farm. Due to transmission pattern and mixing of wild and domestic waterfowl including ducks prevention of the disease through hygiene and biosecurity measure is difficult. In this case hygienic measures and vaccinations would be the very effective tools, as reported elsewhere (Mcferran & Smyth, 2000) for the prevention of EDS-76. Farmers of Bangladesh are practicing EDS-

76 vaccination since long. All commercially vaccines available in Bangladesh are imported and prepared from foreign origin of virus isolates and the vaccines are also costly. The importation of vaccines also exhausts lots of hard earned foreign currency.

For the above mentioned circumstances, preparation and development of a killed EDS vaccine from local isolates was thought to be of great value and enhance financial benefit for the poultry farms of Bangladesh. For vaccine development seed virus was collected from the Department of Microbiology and Hygiene, Faculty of Veterinary Science, Bangladesh Agricultural University (BAU), Mymensingh. The most sensitive indicator system for EDS-76 virus isolation is the embryonated duck eggs (McFerran *et al.,* 1978). They also observed that the EDS-76 virus multiply rapidly and reached higher virus titres in the duck embryos rather than chicken embryos and this indicator system or embryonated duck eggs were used in the present study. In this study; for the preparation of working virus, seed virus was inoculated into 11 days embryonated duck eggs followed by five days of incubation the eggs were chilled at 4˚C and allantoic fluids were harvested. The presence of virus in the allantoic fluids was normally checked by slide HA and micro-titre plate HA tests using chicken erythrocytes. The EDS-76 virus agglutinated only chicken erythrocytes but did not agglutinate the mammalian (buffalo, horse, goat and rabbit) erythrocytes. This feature was in agreement with (Jordan, 1990). The HA titre and EID_{50} of the virus suspension were 15log2 and $log10^{10.6}$/ml. Nabi *et al.,* (1999) used AAF containing EDS-76 with HA titre of 10log2 and the EID_{50} was $log10^{8.4}$/ml for vaccine preparation. Ilyas *et al.,* (2004) prepared an EDS-76 vaccine with AAF having HA titre between 7log2 to 9log2 and the EID_{50} was $log10^{10.88}$/ml. The titre were very much similar compared to the HA and EID_{50} titre used in this study. The initial HA titre was $9log_2$ but after several passage in duck embryo the HI titre increased up to $15log_2$. For the preparation of killed vaccine, the high titred virus suspension was inactivated with formalin. After that, sterility and safety test of the vaccine was performed. A total of 80, 16 weeks aged layer birds were divided into four equal groups and each group consists of 20 birds. The birds of group A and B were vaccinated with experimentally produced BAU-EDS1 and BAU-EDS2 vaccines at a dose of 0.5ml by intramuscular route respectively.

Christensen (1998) described the most susceptible age for EDS-76 to be around 17 weeks. The findings of this study are also similar with the present study. Calnek *et al.* (1991) reported that oil-based EDS-76 virus vaccine induces HI titres up to 256 in unaffected birds while HI titres were up to 16384 in previously exposed birds. Baxendale *et al.* (1980) carried out vaccination trials with formalin inactivated oil adjuvant EDS-76 vaccine (strain BC14); they found the vaccines to be able to protect the birds against natural infection which are closely related with this study. Blood sera sample were collected from birds of all groups before vaccination at 16 weeks of age and post vaccination at 17, 18, 19, 20, 22, 24, 28, 32, 34 and 35 weeks of age. At the age of 32 weeks the birds of all groups were challenged with virulent field isolate of EDS-76 virus.

The antibody titre was measured by HI and ELISA test. Before vaccination the antibody titre was found zero. i.e., there was no antibody found against EDS-76 virus. Ezeibe *et al.* (2008) reported EDS-76 HI titre above 16 was positive for antibody production and protection. The antibody titres

were gradually increased and reached to protective titre ($6\log_2$ is protective of EDS-76 disease Rampin *et al.,* 1980) at 18 weeks. At 18 weeks of age the mean titres of group A, B, C and D were 80, 288, 288 and 00 respectively. The HI titre reached to peak point at 20 weeks of age. Piela and Yates, (1983) found performance of HI and ELISA tests to be mostly same in case of EDS-76 virus; they also found the peak antibody titres approximately at 4 weeks of post vaccination which is very much similar with the present study. At 28 weeks of age the antibody titres were started to decline that's why at 32 weeks of age the challenged was done orally to all birds with live virulent EDS-76 virus. As a result antibody titre again increased at 34 weeks of age after challenge. According to kit literature (Biochek, UK), the birds would be protected against EDS-76 virus if the S/P value is 0.500 or greater and antibody titre above 649 which is similar with the present study. The vaccinated birds responded to the vaccine with increased HI antibodies and lay well throughout the experimental trial period without showing signs of EDS-76. After challenge the average egg production of group A, B, C and D were 85.53%, 91.31%, 92.34% and 64.40% respectively. Das and Pradhan (1992) reported drop of egg production due to EDS-76 ranging between 10.6% and 50.6%. Bishops *et al.,* (1996) observed up to 55% reduction in egg production and bird's having an HI titre ≤16 were susceptible. The egg production was slightly lower with the present study. Raj *et al.* (2004) found ELISA to be 93.6% sensitive and 98.7% specific for EDS-76 virus; the correlation coefficient for ELISA and HI titres was 0.793.

The antibody responses of the birds to experimental vaccination were analyzed. After vaccination HI mean values were determined and it was observed that on 17 to 28 weeks these values were high except control groups. Kumar *et al.* (1988) reported as high as $\log2^{14}$ or more antibody HI titre may be obtained following a vaccination program involving formalin killed vaccine. These results of experimental vaccine were in agreement with (Calnek *et al.,* 1991). Closely similar results were obtained by some researchers (Christensen *et al.,* 1998). A HI titre $\log2^{6}$ is protective of EDS-76 disease (Rampin *et al.,* 1980) and the results of this study of experimental vaccine were much higher than $\log2^{6}$. From the above statement, it can be said that the birds would be protective against EDS-76 disease if challenged after vaccination with the experimental vaccine BAU-EDS1 and BAU-EDS2.

All the vaccines (experimental BAU-EDS1, BAU-EDS2 and Nobilis® EDS) gave satisfactory antibody titre. These finding is in agreement with (Raj *et al.,* 2004). High levels of antibody titre were also obtained by Adair *et al.,* 1986. Prameela *et al.* (2008a) compared ELISA with HI and found both the tests to be sensitive and HI was rapid and simple.

12. Research highlight/findings:

a. The virus was successfully reactivated in duck embryo and HI titre and EID_{50} were determined which were $\log_2$ 14 (1:16384) and EID_{50}-$10^{10.6}$ /ml.

b. The virus sample was successfully inactivated by 0.12% formalin. No growth of bacteria in bacteriological media and no growth of virus in duck embryo indicated the virus to be sterile, completely inactivated and safe to use as vaccine in chicken.

c. Two different experimental EDS vaccines were prepared using two different oil adjuvants and was named as BAU-EDS1 (Montanide oil base) and BAU-EDS2 (Paraffin oil base).

d. Of the two different experimental vaccines BAU-EDS2 induced higher HI antibody titre (576) compared to BAU-EDS1 (448) and the commercial vaccine (448) Nobilis®.

e. After challenge with virulent field isolate of EDS virus egg production in unvaccinated control group was decreased to 47.14% at 4th weeks of post challenge, whereas no change or little decrease in egg production were observed in all the vaccinated groups. However, the average egg production of the groups A (BAU-EDS1), B, (BAU-EDS2), C (Commercial EDS) and D (Control) were 85.53%, 91.31%, 92.34% and 64.40% respectively.

B. Implementation Position

1. Procurement:

Description of equipment and capital items	PP Target		Achievement		Remarks
	Phy (#)	Fin (Tk)	Phy (#)	Fin (Tk)	Completed
(a) Office equipment	27.04.2017	1,00,000	04.12.2017	99,800	
(b) Lab &field equipment	27.04.2017	4,61,800	30.06.2018	4,61,690	
(c) Other capital items	30.06.2018	30,000	11.12.2017 and 12.12.2018	10,000 20,000	

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	6,16,080	5,41,337	5,41,337	00	100%	
B. Field research/lab expenses and supplies	7,26,800	7,19,432	7,19432	00	100%	
C. Operating expenses	61,845	59,481.25	59,481.25	00	100%	
D. Vehicle hire and fuel, oil & maintenance	-	-	-			
E. Training/workshop/ seminar, etc.	-	-	-			
F. Publications and printing	75,000	53,014.75	15,000	38014.75	28.29%	Fund for publication was returned back to BARC
G. Miscellaneous	13640	13640	13640	00	100%	
H. Capital expenses	1,30,000	1,29,800	1,29,800	00	100%	

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)**
1. Development of formalin killed oil adjuvanated EDS vaccine.	1. Virus reactivation in embryonated duck eggs and virus titre determination by micro HA test and EID_{50} 2. Virus inactivation by formalin followed by safety test and sterility test. 3. Preparation of experimental EDS vaccines.	1. The virus was successfully reactivated in duck embryo; HA titre and EID_{50} determined to be $\log_2 14$ and $10^{10.6}$ /ml respectively. 2. The virus sample was successfully inactivated by 0.12% formalin. No growth of bacteria in bacteriological media and no growth of virus in duck embryo indicated the virus to be sterile, completely inactivated and safe to use as vaccine in chicken. 3. Two different experimental EDS vaccines were prepared using two different oil adjuvants and was named as BAU-EDS1 (Montanide oil base) and BAU-EDS2 (Paraffin oil base).	The vaccine could be used to protect layer birds from EDS-76. However, farm level study is needed involving bigger flocks for validation of the vaccine before declaring the vaccine effective and safe to use for

2. Determination of protective efficacy of experimental vaccine against EDS-76 virus in layer birds under experimental conditions.	1. The experimental vaccines and commercial vaccines were inoculated into 16 weeks old layer chicken. 2. Sera samples were collected and antibody titre was determined by HI and ELISA tests. 3. Layer birds were challenged with virulent field isolate of EDS virus in laying hen 4. The egg production in groups after challenge was recorded regularly.	1. Of the two different experimental vaccines BAU-EDS2 induced higher HI antibody titre (576) compared to BAU-EDS1 (448) and the commercial vaccine (448) Nobilis®. 2. After challenge with virulent field isolate of EDS virus egg production in unvaccinated control group was decreased to 47.14% at 4th weeks of post challenge, whereas no change or little decrease in egg production were observed in all the vaccinated groups. 3. The average egg production of the groups A (BAU-EDS1), B, (BAU-EDS2), C (Commercial EDS) and D (Control) were 85.53%, 91.31%, 92.34% and 64.40% respectively.	protecting against EDS-76 virus infection in the commercial layer farms of Bangladesh.
3. Application of vaccine at commercial layer farms.	Could not be performed due to shortage of time and funding.		

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			
Information development			
Other publications, if any		1. One Poster 2. One Oral presentation	1. 11th International Conference of WPSA. 2. 25th BSVER Conference held in 23-24 February, Mymensingh.

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

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

Two types of formalin killed EDS vaccines (BAU-EDS1 and BAU-EDS2) were developed from the local isolates of EDS virus using two different oil emulsions (montanide and paraffin oil respectively). Out of the two vaccines developed BAU-EDS2 performed better and could be transferred to DLS or other for mass production after field trial.

ii. **Generation of new knowledge that help in developing more technology in future**
The knowledge generated on vaccine development may be used for the development of other poultry or livestock vaccines.

iii. Technology transferred that help increased agricultural productivity and farmers' income
None

iv. Policy Support
None

G. Information regarding Desk and Field Monitoring

I). Desk Monitoring:

a) A meeting was arranged by director of BAURES to enhance the research activities
b) An oral presentation was given on the project activities in an Annual workshop arranged by BAURES
c) An oral presentation on a workshop arranged by BARC held on 22 September, 2018
d) Annual report presentation on the project activities in a workshop arranged by BARC held on 24-25 April, 2019

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

a) A team visited on 4th March, 2018 to monitor the research progress
b) A team visited on 7th March 2017 to monitor the research activity

Output: Due to field monitoring, the research activity was enhanced. The research activities were fine tuned according to their suggestion the monitoring team.

Monitoring team visiting the experimental site to observe the progress of the project activity

H. Lesson Learned (if any)

a) The vaccine needs to be tested at commercial scale

b) It is better to use combined ND, EDS and IB vaccines rather than single EDS vaccine to maintain normal production and to avoid vaccination stress.
c) The project needed more time and money. Due to fund deficit and time constraints the activities of the objective-3 could not be started.

I. Challenges (if any)

a) The birds were affected with other diseases that hampered smooth progress of the research activities
b) Due to delayed approval and implementation of procurement plan and release of fund, the project activities were delayed.
c) Sometimes there was unavailability of fertile duck eggs and fertility rate of the eggs was very low which delayed the propagation of virus.
d) Quality of chemicals was not as good as expected.

Signature of the Principal Investigator
Date …….
Seal

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