Project ID 403

## Competitive Research Grant

# Sub-Project Completion Report

## on

Characterization of *Mycoplasma gallisepticum* isolates from Bangladesh and their use in production of diagnostic antigen and development of vaccine candidate

**Project Duration**

**May 2016 to September 2018**

Department of Pathology
Faculty of Veterinary Animal and Biomedical Sciences
Sylhet Agricultural University

Submitted to

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

**September 2018**

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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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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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## Acronyms

CRD= Chronic Respiratory Disease

$^{0}$C= Degree Centigrade

EISA= Enzyme Immuno Slide Assay

*et. al*= *et alii* (Associates)

FAO= Food and Agricultural Organization

HA= Haemagglutination

HI= Haemagglutination Inhibition

i.m.= intrmascular

µl= Microliter

ml= Milliliter

µm= Micro meter

MG= *Mycoplasma gallisepticum*

MS= *Mycoplasma synoviae*

MM= *Mycoplasma meleagridis*

MI= *Mycoplasmas iowae*

PCR= Polymerase chain reaction

PCV= packed cell volume

P-Value= Probability Value

RBC= Red Blood Cell

RDV= Ranikhet Disease Vaccine

rpm= rotation per minute

RSA= Rapid serum agglutination

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# Executive Summary

Different types of Mycoplasmas are present in poultry, among which four are pathogenic that include *Mycoplasma gallisepticum* (MG), *Mycoplasma synoviae* (MS), *Mycoplasma meleagridis* (MM) and *Mycoplasmas iowae*. MG is the most significant pathogen of poultry causing chronic respiratory disease (CRD), which is characterized by rales, nasal discharge, coughing, sneezing, conjunctivitis and air sacculitis with low mortality in uncomplicated cases in chickens. To keep a flock MG free farmers are using live attenuated vaccine containing ts11 and MG 6/85 in layers and breeders with varying results. The imported vaccines are costly and are not readily available. There is extensive pertinent information on mycoplasma in commercial poultry over the entire world but related information for Bangladesh is scarce. Therefore, the research work was undertaken to address the objectives of isolation, identification and characterization of Mycoplasma local isolates and production of diagnostic antigen and vaccine candidates.

For this study, ninety five samples (trachea, lungs) were collected from ninety five (95) poultry farms of sylhet region. Out of the ninety five samples seventeen (17.89%) were found positive for mycoplasma in the selective media (Hayflick Agar and Frey mycoplasma broth). For molecular characterization using PCR assay mycoplasmal DNA was extracted and the assay was conducted for amplification of *Mycoplasma gallisepticum Mgc2 gene*. All seventeen isolates produced bands at 185 bp level with mycoplasma specific primers. From the seventeen PCR positive samples, one sample designated as MG-SAU 1 was selected for the preparation of diagnostic antigen and development of vaccine candidates.

For the preparation of mycoplasma diagnostic colored antigen the selected isolate was inactivated using phenol and stained with Rose Bengal dye. The efficacy of the experimental antigen was evaluated through comparing its detection efficiency with the commercial antigen in slide micro agglutination test of the field samples. Total 150 serum samples were screened by Rapid serum agglutination (RSA) test using experimental and commercial RSA colored antigen. Out of 150 samples 74.6% were found positive with the experimental antigen whereas, using commercial antigen 78.6% of the samples were found positive. The difference of MG detection rate between the experimental and the commercial antigen was found not to be significant statistically at $P>0.05$ level.

Finally, formaldehyde inactivated oil (Montanide ISA70) based *Mycoplasma gallisepticum* vaccine was prepared using the PCR confirmed selected isolate of MG and evaluated in chickens. The amount of immune antigen per dose (0.5ml) of the vaccine was $10^7$ Colony forming units of the bacteria. At the age of 14 days, the broilers were randomly divided into three groups (A, B and C), each having twenty birds. Each of the birds of group A, B and C was then inoculated with 0.5 ml of sterile Frey's broth (negative control), Trial vaccine (TrV) and imported vaccine (ImV) subcutaneously at mid neck region respectively. The birds of group A (Control group) showed 245.35±2.924, 230.95±3.720, 209.3±2.637, 201.9±2.90, 193.8±2.94 mean anti-MG ELISA titer at 0 days of age (Day Old) and 0, 15, 30, and 45 days post vaccination respectively. In contrast, the birds of group B (TrV) showed 244.95± 3.316, 229.85±3.498, 1103.1±6.632, 1621.5±6.447, 2213.75±3.126 serum mean anti-MG ELISA titer at 0 days of age, and 0, 15, 30, and 45 days post vaccination respectively and the birds of group C (ImV) showed 244.8±3.205, 230.35±2.601, 1099.75±5.138, 1545.35±4.416, 2121.75±3.431 serum mean anti-MG ELISA titer on age 0 day , and 0, 15, 30, and 45 days post vaccination respectively. The oil based MG trial vaccine could induce protective level of anti-MG-ELISA antibodies in broilers that reached maximum at 45 days post priming (2213.75±3.126) which again was comparable with the imported vaccine (2121.75±3.431). The oil based vaccine again did not interfere with the maternal antibody titer of the birds so, it was concluded that the preparation could be used as vaccine candidate in the future study.

# CRG Sub-Project Completion Report (PCR)

## A. Sub-project Description

1. **Title of the CRG sub-project:**
   Characterization of *Mycoplasma gallisepticum* isolates from Bangladesh and their use in production of diagnostic antigen and development of vaccine candidate

2. **Implementing organization:**
   Department of Pathology and Department of Microbiology & Immunology
   Sylhet Agricultural University

3. **Name and full address with phone, cell and E-mail of PI/Co-PI (s):**
   PI: Dr. Monira Noor
   Associate Professor
   Department of Pathology
   Faculty of Veterinary Animal and Biomedical Sciences
   Sylhet Agricultural University
   Sylhet 3100, Bangladesh
   Cell: +880 1920-294699, E-mail: monira_path@yahoo.com
   CO-PI: Dr. Sultan Ahmed
   Professor, Department of Microbiology & Immunology
   Faculty of Veterinary Animal and Biomedical Science
   Sylhet Agricultural University
   Sylhet 3100, Bangladesh
   Cell: +880 1711 140389, E-mail: drsultanahmed@yahoo.com

4. **Sub-project budget (Tk):**
   4.1 Total: Tk. 17,00000.00
   4.2 Revised (if any):

5. **Duration of the sub-project:**
   5.1 Start date (based on LoA signed): 10th july, 2017
   5.2 End date: 30 September 2018

6. **Justification of undertaking the sub-project:**
   *Mycoplasma gallisepticum* (MG) causes chronic respiratory disease in domestic poultry, especially in the presence of management stresses and/or other respiratory pathogens. Without proper diagnostic and preventive measures, a case of MG infection could cause huge economic loss of a farmer. Up to now, only limited information is available on the identification and molecular characterization of Bangladeshi MG isolates. False positive results often arise when commercially available imported diagnostic kits are used. Immuno prophylaxis failure is due to difference in antigen variation. In addition, production of biologics using indigenous isolates of MG has not yet been initiated, and the high cost and poor availability of MG biologics in the country has limited their use by poor farmers. These circumstances have helped to ever existing mycoplasmosis in Bangladeshi poultry flocks. With the advent of multi-age commercial layer complexes, control by vaccination became indispensible.

**7. Sub-project goal:**

To control mycoplasmosis caused by *Mycoplasma gallisepticum.*

**8. Sub-project objective (s):**

a. To characterize *Mycoplasma gallisepticum* field isolates.
b. To prepare diagnostic antigen.
c. To develop vaccine candidate from the field isolate.

9. **Implementing location (s):**

The study was conducted at the Department of Pathology, and the Department of Microbiology and Immunology, Faculty of Veterinary Animal and Biomedical Sciences, Sylhet Agricultural University, Bangladesh

**10. Methodology in brief:**

The handling of animals in the study was performed in accordance with the current Bangladesh legislation (Cruelty to Animals Act 1920, Act No. 1 of Government of the People's Republic of Bangladesh). The specific experiments were approved by the Ethics Committee of Sylhet Agricultural University, Bangladesh.

**10.1. Isolation, identification and characterization of *Mycoplasma galisepticum***

**Collection of tissue sample, processing and isolation of the organism**

Tissue samples such as lungs, air sacs, and trachea from gross post mortem lesions of naturally occurring mycoplasma suspected poultry were collected aseptically from 95 (ninety five) different poultry farms of Bangladesh. Then the tissue samples were crushed using mortar and pestle and homogenous tissue suspension were passed through sterile sieve and filter paper. The filtrate was then centrifuged at 5000 rpm for 20 minutes and the supernatant were passed though sterile bacterial membrane filter paper of the pore size of 0.45µm (the size of mycoplasma ranges from 0.25 to 0.45µm). Finally, the filtrate was dispensed (inoculated) on plates of Hayflick's agar 25 and broth media containing supplement that can prevent the growth of bacteria and fungus and incubated at under microaerophilic condition (5% CO2). The plates were examined daily for any visible colony growth under stereomicroscope (25×). Prominent and visible characteristic *Mycoplasma* (round colonies with central nipple) colonies were incised including the agar and sub-cultured on to Mycoplasma agar and broth to get pure colonies. After obtaining pure culture, biochemical tests such as fermentation of glucose was done as preliminary biochemical identification of *Mycoplasma.*

**Detection/identification of MG DNA by conventional PCR**

One micro liter broth culture of the MG isolates was used for DNA extraction by using FavorPrep™ Blood Genomic DNA Extraction Mini Kit as per manufacturer's instructions. For confirming the presence of MG, the DNA extracted samples was processed for polymerase chain reaction (PCR) using MG specific primer pairs. The master mix comprised of RNase free water-5µl, 10 × PCR Buffer-5.00µl, dNTP (l0mM)- 1.00µl, F Primer MG-14F: 5'-GAG-CTA-ATC-TGT-AAA-GTT-GGT C-3' (5pmole/µl)- 2µl, R Primer MG-13R: 5'-GCT-TCC-TTG-CGG-TTA-GCA-AC-3' (2pmole/µl)- 2µl, Taq DNA polymerase (5 U/µl)- 0.50µl, and MgCl2 (25mM)- 1.50µl, Template (DNA)- 3µl (ISO/IEC 17025:2005 NVI-QMS SOP).

Amplification of the *mgc2* gene was performed using Positive and negative controls and 45µl volume of the reaction mixture was dispensed into each PCR tube. The tubes were then taken to another clean area where the appropriate DNA sample (5µl) was added to each tube. Positive and negative controls were used in each run. The tubes were then placed in a thermal cycler (Applied Biosystem 2720) for the following cycles: initial denatutation at 94°C/5mints for one cycle, 40 cycles: 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds, 1 cycle (final extension): 72°C for 5 minutes and stored at 4°C (OIE, 2012; ISO/IEC 17025:2005 NVI-QMS SOP). Amplified PCR products were detected using conventional 2% agarose gel electrophoresis.

**10.2. Preparation of mycoplasma diagnostic antigen from local isolate**

The local isolate of *Mycoplasma gallisepticum* was transferred to a conical flask containing 50 ml of *Mycoplasma* broth base and incubated for 72 hours at 37$^{o}$C in 10% CO2. After incubation, there was dense growth of the organism in the culture broth and the color of media was changed from red to yellow. The organisms were pelleted by centrifugation at 14000 rpm for 20 minutes and supernatant was discarded. The pellet was washed with PBS thrice to remove the traces of media. One percent packed cell volume (PCV) was adjusted with the help of hopkin's tube. This 1% PCV antigen was again pelleted by centrifugation. The pellet was re-suspended in phenol saline (20 ml). Rose Bengal dye (1 %) was added in dense culture (0.5%) and the culture was again incubated at 37$^{o}$C in 10% CO2 for 24 hours. Phenol (0.5%) was added in the stained culture, and the culture was incubated again for 2 hours at 37$^{o}$C for inactivation. The inactivated stained antigen was centrifuged at 14000 rpm for 15 minutes. The supernatant was discarded. The pellet was washed thrice in phenol saline to remove the residues of media and stain, in final suspension the packed cell volume (PCV) was adjusted to 1% by Hopkin's centrifuge tube. The stained antigen was homogenized thoroughly by vortexing and was kept for future use as rapid slide agglutination (RSA) test antigen.

**Evaluation of RSA colored antigen**

The RSA antigen prepared from local isolate was then evaluated by doing slide micro agglutination tests of the field samples. *Mycoplasma gallisepticum* positive and negative hyper immune sera were used as control and results of locally prepared RSA antigen were compared with commercial RSA antigen (Charles River, USA). Test was performed according to the Commercial RSA antigen manufacturer guideline (Charles River, USA). All the components of the test including test sera, colored antigen (commercial and prepared from local isolate) positive and negative controls and slides were pre warmed at room temperature. Antigen was homogenized by vortexing for few seconds. Serum samples (n=150) were screened by RSA test using local and commercial RSA colored antigen and results were noted.

**10.3. Preparation of mycoplasma killed vaccine**

For the development of MG vaccine, PCR confirmed positive local isolate was used as seed. The active MG seed was inoculated @ 10% v/v into Frey's broth and incubated at 37$^{0}$C with 10% CO2 tension and observed daily for any growth. After 48 hrs of incubation the cultured media tubes was taken out of incubator and 10 ml of growth suspension was transferred to graduated Hopkin's centrifuge tube and centrifuged at 3000xg for 20 min to estimate packed cell volume per ml of the media. The mass of MG antigen was adjusted to 1% in the Hopkin's tube using phosphate buffered saline (pH 7) as diluent. For inactivation of the growth of MG 0.125% of 37% formaldehyde was used and the tubes were incubated at 37$^{o}$C with 10% CO2 tension for providing 12 hrs of interaction time. To ascertain the completion of inactivation, the inactivated broth were separately cultured on Frey's media and broth, incubated at 37$^{o}$C with 10% CO2 tension and observed for seven days for the

appearance of any specific growth of *Mycoplasma* colonies or color change respectively. After confirmation of growth inactivation the montanide oil adjuvant was admixed at 4:1 ratio with the inactivated MG to properly emulsify the mycoplasmal biomass. The inactivated culture was processed further according to Biro *et al*. (2005) to ensure the sterility and safety of the culture.

**Sterility test**

The experimental oil adjuvant vaccine was sterilized by heating at 160°C for 1 hour (OIE; 2012). Sterility tests were done in sterility media such as, Triptic soy broth, Tryptose agar and Sabroud agar with incubation at $37^0$C for 7 days and no growth of any organism confirmed the sterility of the vaccine.

**Safety test**

One ml (double of the field dose) of the trial vaccine was inoculated subcutaneously at mid neck region into six chickens. The chickens were kept under observation for 14 days.

**Evaluation of MG vaccine efficacy**

Sixty (one-day-old) broilers were reared under standard management conditions. The feed and fresh water were provided *ad libitum* during whole period of experiment. Routine vaccination program for broiler chicken was also implemented. All the chickens were tested for serum antibodies against MG at 0 days of age through indirect ELISA kit prior to inoculation of both the oil based vaccines (experimental and imported vaccine). At the age of 14 days, the broilers were randomly divided into three groups (A, B and C), each having twenty birds. Each of the birds of group A were inoculated with 0.5 ml of sterile Frey's broth subcutaneously and kept as negative control (NC). Each of the birds of group B and C were injected with 0.5 ml of trail vaccine (TrV) and imported vaccine (Imv) respectively through subcutaneous (s/c) route at mid neck region. Chickens in all groups were monitored daily up to 60 days of experiment and observations were recorded for their general conditions, clinical signs of the disease or any mortality. Blood samples (1 ml) were collected from jugular vein of each bird from each group on 14, 30, 45 and 60 days of age i.e., 0, 15, 30 and 45 days post vaccination. The serum from each of the blood samples were separated and transferred to properly labelled serum vials and stored at $-20^0$C till further processing. Anti MG ELISA antibody titer of each serum samples were determined through ELISA kit. The data were analyzed to compare the antibody response of the birds to local and imported vaccines. No challenge test was performed

**Data analysis**

All data were processed using MS Excel and SPSS software.

## 11. Results and discussion:

### 11.1. Isolation, identification and characterization of *Mycoplasma galisepticum*

For isolation of mycoplasma total 95 (ninety five) tissue samples were collected from 95 poultry farms of sylhet and Dhaka division. Out of the 95 samples only seventeen (17.89%) were positive in the mycoplasma specific media (Figure 1-3). Primary isolates with prominent *Mycoplasma* fried egg like micro colonies were examined under stereomicroscope (25× magnification) after three to four days of incubation. Pure *Mycoplasma* micro colonies were obtained after five to six subsequent sub-culturing onto agar plates (Figure 4). In glucose fermentation test, all the isolates fermented glucose which was indicated by color change from red to yellow (Figure 5). For molecular identification of *Mycoplasma gallisepticum* DNA was extracted from broth culture (Figure 6). Among the seventeen isolates, 12 (70.58%) were found strong positive and 5 (29.41%) were weak positive to MG that was

evidenced by the migration of PCR amplified DNA products (bands) at 185 bp position along with the positive control (Figure 7). Mycoplasmas are fastidious organisms. However, it is suspected that either the widespread use of antibiotics in the flocks sampled or the difficulty of culturing the mycoplasma organism could have kept the number of positive samples down.

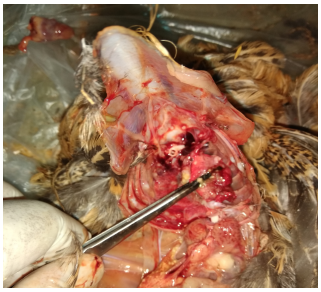

**Figure 1:** Collection of samples for mycoplasma isolation

**Figure 2:** Sample for suspected of mycoplasma infection

**Figure 3:** Inoculation of sample onto media for isolation of mycoplasma

**Figure 4:** Fried egg colony on Hayflick agar


**Figure 5:** Glucose fermentation test. Positive samples shows color changes from red to yellow.

**Figure 6:** Preparation for DNA extraction

From among the seventeen positive isolates one isolate was designated as MG-SAU 1 which was used for the production of RSA colored antigen and vaccine candidate preparation.

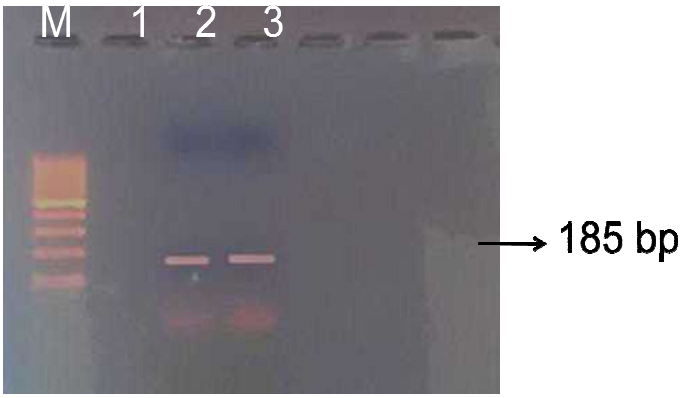


**Figure 7**: Confirmation of mycoplasma isolate by PCR
*M- marker, 1- water control, 2- positive control, 3- mycoplasma field isolate.*

### 11.2 Preparation and evaluation of RSA colored antigen from local isolates

The Rose Bengal dye-stained *Mycoplasma* antigen showed pink color and the agglutination result was easily seen (Figure 8). Out of 150 samples tested 112 (74.6%) samples were positive and 38 (25.3%) were negative with locally prepared antigen, whereas with commercial antigen 118 (78.6%) samples were positive and 32 (21.3%) were negative (Table 1). Similar results have also been reported by Arefin *et al*. (2012) and Rassol *et al*. (2017). The difference between the commercial and locally prepared RSA antigens in the detection of MG was found not to be statistically significant at P>0.05 level. It was therefore concluded that RSA colored antigen prepared from local isolates could be used for rapid screening of MG in poultry flocks.

**Table 1:** Comparison of the efficacy of locally prepared and commercial RSA antigens for detection of antibodies against *Mycoplasma gallisepticum*

| Antigen | No. of samples | Positive | Negative | P value |
|---|---|---|---|---|
| **Locally prepared** | 150 | 112 (74.6%) | 38 (25.3%) | P>0.05 |
| **Imported** | 150 | 118 (78.6%) | 32 (21.3%) | |

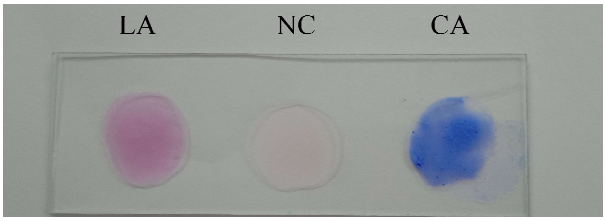


**Figure 8.** RSA test using locally prepared and commercial colored antigen
*LA: locally prepared antigen, NC: Negative control and CA: Commercial antigen*

### 11.3 Preparation of formalin killed mycoplasma vaccine

The local isolate (*Mycoplasma gallisepticum*) grew well in Frey's broth within 24 hours at $37^0$C with 10% CO2 showing $10^7$ colony forming units (CFU) per ml of the medium. The mycoplasma packed cell was formed after centrifugation and was inactivated using formaldehyde. Similar procedure was performed by other researcher as they used 37%: Merck @ 0.05% (Koski *et al*., 1976; Rimler *et al*., 1978; Hildebrand *et al*., 1983; Glisson *et al*., 1984; Glisson and Kleven, 1985; Sasipreeyajan *et al*., 1985; Yagihashi *et al*., 1987). Formaldehyde molecules bind with amine group of amino acids in protein molecule of the organisms and thus inactivate its viability. Higher concentration of the inactivant may reduce antigenicity of the organisms.

**Safety and sterility test**

The results of sterility test indicated that the prepared inactivated MG vaccine is free from any contaminants (aerobic bacteria, anaerobic bacteria, mould and yeast). Concerning safety of the prepared vaccine, it was found that chickens vaccinated with even double field vaccine dose didn't show any abnormalities or adverse reaction. As regards to sterility and safety tests, the prepared vaccine cover all conditions described previously (OIE, 2012).

**Evaluation of inactivated vaccine candidate**

The oil based MG vaccine induces detectable level of anti-MG-ELISA antibodies. The oil based MG inactivated vaccines (Group A- control, Group B- local vaccine and Group C- imported vaccine) were inoculated in broiler chickens. At day old the mean antibody titer of group A, B and C were 245.35±2.924, 244.95± 3.316 and 244.8±3.205, respectively. Broilers from all the groups were vaccinated at 14 days of age. The broilers vaccinated with trail vaccine (TrV) showed 229.85±3.498, 1103.1±6.632, 1621.5±6.447 and 2213.75±3.126 serum mean anti-MG ELISA titer on 0, 15, 30, and 45 days post vaccination respectively. The imported vaccine (ImV) group showed 230.35±2.601, 1099.75±5.138, 1545.35±4.416 and 2121.75±3.431 serum mean anti-MG ELISA titer on 0, 15, 30, and 45 days post vaccination respectively. However, non-vaccinated birds (Control group) showed 230.95±3.720, 209.3±2.637, 201.9±2.90 and 193.8±2.94 mean anti-MG ELISA titer on 0, 15, 30 and 45 days post vaccination respectively (Table 2). According to the ELISA kit used the MG ELISA antibody titer ranging 744 or greater gives 100% protection. All 3 groups have similar antibody titer at day 0. After vaccination mean antibody titer of Group B and C was found to be increased significantly whereas in group A mean antibody titer was decreased gradually. Similar type of results were also found by Gondal *et al.*, 2013, Jacob *et al*., 2014, and Yoder and Hopking, 1985.

**Table 2:** Comparative antibody response in broilers with local and imported MG vaccines

| **Age (days)** | **ELISA Antibody Titer** | | | **significance** |
|---|---|---|---|---|
| | **Group A Control** | **Group B Local vaccine** | **Group C Imported vaccine** | |
| **0 (day old chick)** | 245.35±2.924 | 244.95± 3.316 | 244.8±3.205 | P<0.001 |
| **14 (0 day of vaccination)** | 230.95±3.720 | 229.85±3.498 | 230.35±2.601 | |
| **30 (15 days post vaccination)** | 209.3±2.637 | 1103.1±6.632 | 1099.75±5.138 | |
| **45(30 days post vaccination)** | 201.9±2.90 | 1621.5±6.447 | 1545.35±4.416 | |

| | | | | |
|---|---|---|---|---|
| **60 (45 days post vaccination)** | 193.8±2.94 | 2213.75±3.126 | 2121.75±3.431 | |

Anti-MG ELISA titer in day old chicks indicates that the breeder flock was either vaccinated against MG or carrier of MG infection. Moreover, killed vaccine did not interfere the maternally derived anti-MG ELISA antibody titers. Use of MG bacterin in chickens is a common practice to protect them against respiratory signs, airsacculitis, egg production losses and reducing egg transmission (Rimler *et al.*, 1978; Hildebrand *et al.*, 1983; Glisson *et al.*, 1984; Sasipreeyajan *et al.*, 1985; Sasipreeyajan *et al.*, 1987; Yagihashi *et al.*, 1987). The major advantage of oil-emulsion bacterin is that protection against economic losses can be obtained without the introduction of a live-vaccine strain. This study concludes that Oil based MG bacterin (killed MG vaccine) protects infection from MG and thus could be used as vaccine candidate in the future study.

**12 Research highlight/findings:**

- Among the 95 samples collected from mycoplasma affected farms 17 (17.89%) were found positive for mycoplasma by culture characteristic.
- All of the 17 samples were also positive in PCR assay with *Mycoplasma gallisepticum* specific primers.
- For rapid serum agglutination (RSA) test the Rose Bengal dye-stained mycoplasam colored antigen was prepared using local isolate (designated as MG-SAU 1).
- Among the 150 serum samples subjected to RSA test using locally prepared antigen and commercial antigen 74.6% and 78.6% were detected to be positive respectively. The difference of MG detection rate between the experimental and the commercial antigen was found not to be significant statistically at P>0.05 level.
- Mycoplasma formalin killed vaccine was prepared from MG-SAU 1. After vaccination (at 45 days post vaccination) with locally produced vaccine $\log_{10}$ antibody titer was found to reach at maximum protection level with anti-MG ELISA titer of 2213.75±3.126 that was comparable with the commercial vaccine (2121.75±3.431).

**B. Implementation Position**

**1. Procurement:**

| Description of equipment and capital items | PP Target | | Achievement | | Remarks |
|---|---|---|---|---|---|
| | Phy (#) | Fin (Tk) | Phy (#) | Fin (Tk) | |
| (a) Office equipment | Printer, Wooden shelf, Computer table ,Computer chair | 37500.00 | Printer, Wooden shelf, Computer table ,Computer chair | 37500.00 | |
| (b) Lab &field equipment | Steriomicroscope, Thermomixer, ELISA kit, PCR mastermix, DNA extraction kit, Hayflicks agar base, PPLO agar | 575000.00 | Steriomicroscope, Thermomixer, ELISA kit, PCR mastermix, DNA extraction kit, | 575000.00 | |

| | | | | | |
|---|---|---|---|---|---|
| | base, PPLO broth base, Freys broth base, Freys agar base, PCR primer, Agarose gel, TAE buffer, RNASE free water, Growth supplement,Vortexer | | Hayflicks agar base, PPLO agar base, PPLO broth base, Freys broth base, Freys agar base, PCR primer, Agarose gel, TAE buffer, RNASE free water,Growth supplement,Vortexer | | |
| (c) Other capital items | Refrigerated centrifuge Machine, Gel electrophoresis | 400000.00 | Refrigerated centrifuge Machine, Gel electrophoresis | 400000.00 | |

**2. Establishment/renovation facilities:**

| Description of facilities | Newly established | | Upgraded/refurbished | | Remarks |
|---|---|---|---|---|---|
| | PP Target | Achievement | PP Target | Achievement | |
| For the identification of poultry pathogen | Established modern diagnostic laboratory | 100% | | | |

**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 | 318790 | 318790 | 318790 | 0 | 100% | |
| B. Field research/lab expenses and supplies | 718710 | 687639 | 688829 | -1190 | 90% | Could not pay tax /vat due to insufficient GoB fund |
| C. Operating expenses | 115000 | 93013 | 92668 | 345 | 98% | 345 taka for bank |

| Items of expenditure/activities | Total approved budget | Fund received | Actual expenditure | Balance/ unspent | Physical progress (%) | Reasons for deviation |
|---|---|---|---|---|---|---|
| | | | | | | closing charge |
| D. Vehicle hire and fuel, oil & maintenance | 0 | 0 | 0 | 0 | 0 | - |
| E. Training/workshop/ seminar etc. | 0 | 0 | 0 | 0 | 0 | - |
| F. Publications and printing | 85000 | 43074 | 13050 | 30024 | 25% | Unspent money was returned |
| G. Miscellaneous | 25000 | 24366 | 24366 | 0 | 100% | - |
| H. Capital expenses | 437500 | 437500 | 437500 | 0 | 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)** |
|---|---|---|---|
| Characterization of mycoplasma field isolates | i. Sample collection, isolation and identification of field isolates<br>ii. Microbial culture and PCR assay | Seventeen (17) isolates of *Mycoplasma gallisepticum* obtained and confirmed by PCR assay | RSA colored antigen of MG will help in rapid diagnosis of mycoplasma at the field level. The vaccine that has been developed, although need further trial at the field level, will help controlling the disease in the poultry farm that in turn will help reducing the loss from the disease. |
| Preparation of diagnostic antigen | i. Inactivation of organisms in phenol and preparation of colored antigen using Rose Bengal dye<br>ii. Evaluation of the antigen through using in RSA test and comparison of the test with the commercial antigen | Rose Bengal dye-stained mycoplasam colored antigen prepared from local isolate showed more or less the same efficacy (with a detection rate of 74.6%) as compared with the commercial antigen (78.6%) | |
| Development vaccine candidates | Inactivation of Mycoplasma using formaldehyde and mixing with oil adjuvant for the development of vaccine Evaluation of vaccine on animal and comparison with the commercial vaccine | Formalin killed mycoplasma vaccine prepared using the local isolate showed the same efficacy (with anti-MG ELISA titer of 2213.75±3.126) as compared with the commercial vaccine (2121.75±3.431). | |

**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 | | Under process |
| Information development | | | |
| Other publications, if any (MS thesis) | 1 | | Under process. |

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

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

Mycoplasma colored antigen for rapid serum agglutination (RSA) test to detect mycoplasma infection in the affected poultry farms.

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

Mycoplasma vaccine candidate from local isolates developed that in future could be used for the development of an effective vaccine to combat mycoplasmosis in poultry.

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

| Date of workshop/ meeting | Description of Workshop/meeting | Output |
|---|---|---|
| 21.12.2017 | Research Review Workshop on CRG sub-projects at BARC auditorium | Discussed the implementation progress of CRG activities. |
| 24.04.2018-25.04.2018 | Progress workshop of CRG sub projects under Livestock Division, BARC | Discuss the progress of CRG activities |
| 15.05.2018-16.05.2018 | Monitoring Workshop on CRG sub-projects | Discuss the Monitoring report of implementation and progress of CRG activities by Tchnical subdivision, BARC |
| 22.09.2018-23.09.18 | annual workshop on CRG sub-project | Discuss the annual progress of CRG activities |

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

| Date of visit | No. of Visit | Team | Output |
|---|---|---|---|
| 05.04.2018 | 1 | Technical Division/ Unit, BARC | |
| 7.04.2018 | 1 | PIU-BARC, NATP-2 | |
| 4.06. 2018 | 1 | Internal monitoring | |

**H. Lesson Learned (if any)**

For successful implementation of Mycoplasma vaccine production project needed more time than it was allocated.

**I. Challenges (if any)**

Keeping pace with the time was a big challenge.

Signature of the Principal Investigator
Date …….
Seal

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

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