



EVALUATION OF BIVALENT INACTIVATED INFECTIOUS BRONCHITIS VIRAL VACCINE PREPARED FROM LOCAL ISOLATES

Reda, R. Fathy^{*1}, Magda, M.A.Moustafa¹, Zyan, K.A¹, El boraay, I.M¹, Susan, S. El-Mahdy²

¹ Department of Avian and rabbit diseases, Faculty of Veterinary Medicine, Benha University, Egypt.

² Central Lab. for Evaluation of Vet. Biologics, Absassia, Cairo, Egypt

* Corresponding author: Reda, R. Fathy; email: reda.megahed@fvmt.bu.edu.eg

ABSTRACT

Infectious Bronchitis (IB) is currently one of the most important diseases in poultry flocks all over the world causes huge economic losses in poultry industry. Many IBV outbreaks associated with respiratory distress, nephropathy, and high mortalities were attributed to the circulation of new nephropathogenic IBV variant 2 strains. This study was conducted to development of bivalent inactivated IBV vaccine by using the local classical and variant isolates (KP279995/2014 and KP279998/2014 respectively) which isolated from poultry farms in Egypt as previous surveillance study during IBV outbreak from Al-Sharkia and Al-Qalubia governorates. The locally prepared vaccine formulated by using formalin for inactivation and Montanide™ ISA71 RVG as oil adjuvant. The prepared bivalent inactivated (IBV) vaccine was tested for sterility, safety and potency, the efficacy of the prepared vaccine was applied in specific pathogen free chicks (SPF) for monitoring the antibody titers by using of Enzyme Linked Immuno Sorbent Assay (ELISA) post vaccination with estimation of shedding parameters by Real-Time RT-PCR in challenging birds with 10^{4.5}EID₅₀/dose challenge IBV strains (classical and/or variant). The results revealed that the prepared vaccine free from any bacterial or mycotic contamination also safe after double dose inoculation in SPF chicks. The efficacy of inactivated vaccine showed from 92% to 96% protection against homologous challenge based on assessment parameters. This confirms that poultry industry can be protected from IB disease if using locally isolates in preparing of inactivated vaccine which reduce the economic losses caused by IB infection viruses in Egypt.

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INTRODUCTION

The avian infectious bronchitis virus (IBV) is a highly contagious pathogen of commercial chickens with a predilection for the upper respiratory tract and it cause reduction in production, in addition to causing disease in kidneys resulted in nephritis .In adult, IBV effect on reproductive tract and produce irreparable damage to oviduct and production of abnormal eggs. (Cook *et al*, 2012; Jackwood, 2012; Jackwood and De Wit, 2013).

Infectious bronchitis virus IBV is a gammacoronavirus, family coronaviridae, order Nidovirales , the virus is a single-stranded, positive sense, 27 kb RNA genome that encodes many nonstructural proteins involved in replication, three

major structural proteins (spike (S), envelope (E), membrane glycoprotein (M) involved in virion formation and a protein involved in genome packaging nucleocapsid (N) (Cavanagh, 2007) .

The continuous mutation and recombination events and the emergence of novel IBV variants urge researchers to establish a simple phylogeny-based classification system. In Egypt, Massachusetts D3128, D274, D08880, 793B, variant 1 and 2 strains (Abdel-Moneimet *et al*. 2006; Zanaty *et al.*, 2016) and IS/1494/06 nephropathic IBV strain are frequently isolated from poultry (Susan *et al*; 2010). Currently, the IBV variant 2 is the most predominant serotype in Egypt, causing massive losses in broiler, layer, and breeder sectors (Susan *et al*. 2011; Abdel-Moneim *et al*. 2012).

Vaccination remains the main control approach of IBV infection. However, the continuous genetic, antigenic, and tissue tropism changes of the circulating IBV caused continual vaccine failure events (Cook *et al.* 2012). IBV vaccines are based on live attenuated or killed vaccines derived from classical or variant serotypes. In Egypt, Mass-type and variant vaccine strains were employed to provide broader protection in poultry. The lack of cross protection between imported vaccine strains and field strains may explain the failure to establish an effective vaccination program against IBV (Kim *et al.* 2013; Toro *et al.* 2015).

Emergence of new “variant” strains require rapidly preparation of inactivated autogenous vaccines for controlling IB in laying birds without the risks of using a live variant vaccine that could spread and potentially produce the disease. Inactivated variant vaccines may offer better protection against challenge with the virulent variant IBV than inactivated vaccines containing standard serotypes such as Mass and Conn (Ladman *et al.* 2002; Jackwood, 2012). The aim of the present study was directed to develop and evaluate inactivated vaccine from IBV strains currently circulating in the Middle East including an Egy/variant 2 strain belonging to the unique Middle East lineage and classical strain also assessment of quality control of this locally prepared inactivated IB vaccine.

MATERIALS AND METHODS

1. IBV strains

IBV strains were previously isolated from different localities by Reda *et al.* (2015), (Chicken / Egypt KP279995 / VACSERA / 2014) (classical) and (Chicken / Egypt KP279998 / VACSERA / 2014) (variant) The two strains were propagated in SPF-ECE and tittered with infectivity titer 10^{8.5}EID₅₀ / dose as viral seed for preparation of bivalent inactivated IB vaccine and challenge dose was adjusted to be 10^{4.5} EID₅₀ /ml, the titration was calculated according to Reed and muench, (1938).

2. Experimental hosts

2.1. Embryonated Chicken Eggs (SPF-ECE)

Fertile specific pathogen free embryonated chicken eggs (SPF- ECE) were purchased from the SPF egg farm, Kom Oshim, EL-Fayoum Governorate, Egypt. The eggs were used for propagation and titration of the seed IB viruses and assurance of complete virus inactivation of tested inactivated IB vaccine.

2.2. SPF chicks

Total number of 200, one day-old SPF chicks were purchased from SPF poultry project, Kom Oshim, EL-Fayoum Governorate, Egypt. All birds were housed

in a separated negative pressure –filtered air isolators and all experimental procedures were reviewed and approved by the Ethical and Animal Welfare Committee.

3. Enzyme –linked Immunosorbent Assay (ELISA) kit

Serum samples were tested for detection of antibodies to IBV after vaccination by using ELISA Kit which was obtained from Biocheck poultry immune assays. IBV antibody test kit (CK119) with Serial No. F69371 product code: 6020 used in this study, all procedures were conducted according to manufacturer's instructions.

4. Real-time Reverse transcriptase chain reaction (RRT-PCR)

Tracheal and kidney samples were collected and stored at -20°C till used , it were tested for detection and titration of IBV in vaccinated and un vaccinated control groups at 3th,5th ,7th days post challenge . Extraction of viral RNA carried out by QIA amp Viral RNA Mini Kit (QIAGEN) with catalogue No. (52904). All procedures were conducted according to manufacturer's instructions. Preparation of master mix was conducted using Quanti Tect probe RT-PCR with catalogue no. (204443) provide accurate real-time quantification of RNA targets, Primers and probes used were supplied from Metabion (Germany) as shown in table (1). The numbers of viral genome copies were quantified in a TaqMan® real time RT-PCR targeting IBV gene.

Table 1: accurate real-time quantification of RNA targets, Primers and probes

| Virus | Gene | Primer/ probe sequence 5'-3' |
|-------|------|---|
| IB | N | AIBV-(Forward) ATGCTCAACCTTGTCCTAGCA |
| | | AIBV-(Reverse) TCAAAGTGC GGATCATCACGT |
| | | AIBV-TM (Probe) [FAM]TTGGAAGTAGAGTGACGCC CAAAGTTC A [TAMRA] |

Meir *et al.* (2010)

Preparation of an experimental batch of bivalent inactivated IBV vaccine

1. Propagation and titration of IB viruses in SPF-ECE: OIE, (2018)

Two field isolates (KP279995, Classical) and (KP279998, Variant) were propagated in the allantoic fluid of SPF-ECE at 9-11 day old and incubated at 37°C with 80% humidity, after 72 hrs of inoculation, allantoic fluids were harvested from about 12 passages of inoculation. The second step was titration in SPF-ECE (tenfold serial dilution in sterile physiological saline, 0.1ml of the virus suspension dilution was inoculated in to the allantoic sac of each of 10 days old SPF-ECE and incubated at 37°C with daily candling.

2. Inactivation of the propagated IBV

The harvested infected allantoic fluid was treated with Formalin 37 % (Sigma- Germany) at a final concentration of 0.1 % with continuous stirring during inactivation process according to **Beard, (1989)**. Samples from the inactivated virus, should be tested for completion of inactivation by passage (at least 2 blind passages) in to 9-11 days old, ECE (0.1 ml/egg) via the allantoic cavity route and examined for three days.

3. Preparation of the vaccine emulsion

It was prepared as water in oil emulsion (W/O) using Montanide™ ISA71 RVG (SEPPIC, Paris La Defense. 92806 Puteaux, France batch No. T21931) at a ratio of 30/70 (v/v) according to the standard protocol of SEPPIC for manufacture instruction **BenArous et al. (2013)**.

Evaluation of prepared bivalent inactivated IBV oil emulsion vaccine

Testing of quality control of the prepared bivalent inactivated IBV vaccine including sterility, safety and potency tests which were carried out according to **OIE, (2018)** and **Egyptian standard regulation for veterinary Biologics, (2017)**.

1-Sterility test:

Media used for sterility test:

1-Nutrient agar media for detection of aerobic bacterial contamination.

2-Thioglycolate broth for detection of anaerobic bacterial contamination.

3-Saburaoud dextrose agar media for detection of fungal contamination.

It was applied to confirm that the prepared bivalent IBV inactivated vaccine was free from bacterial and fungal contamination. Samples from the tested vaccine inoculated into nutrient agar and thioglycolate broth media then incubated at 37°C for detection of any bacterial contamination also the sample were cultured on Saburaoud dextrose agar

media and incubated at 25°C for 14 days to detect any fungal contamination. The inoculated media were inspected daily for any possible growth.

2-Safety test

Forty SPF chicks aged three weeks old were divided into two equal groups (20 chicks/ group), the first group was inoculated S/C with double the field dose of the tested vaccine and the second group were kept unvaccinated as control. Birds in both groups were observed for any signs of local reaction or appearance of any clinical signs of IBV and post mortem examinations to detect any pathological lesions for 15 days.

3-Potency of the prepared vaccine

A total of 160 SPF one day old chicks were used to evaluate the efficacy of locally prepared bivalent inactivated IB vaccine, they were housed in brooder units within isolation facilities. At 21 days of age, the chicks were divided into six equal groups (25 birds/group) and one control negative group (10 birds) as in table (2). Frist three groups vaccinated S/C with field dose (0.5ml) of prepared vaccine / bird; the other three groups were left without vaccination. Blood samples were taken weekly for serological analysis of IB antibody level using ELISA test. Three weeks post vaccination, the vaccinated groups (G1, G2, G3) and unvaccinated control groups (G4, G5, G6) were subjected for challenge test with IBV strains and group (7) kept as control (-ve) unvaccinated unchallenged. Each bird received a dose of 0.1 ml oculonasal rout of IBV strains ($10^{4.5}$ EID₅₀/ml) and observed for 15 days after challenge for clinical signs and postmortem lesion in trachea and kidney which were collected at 3rd, 5th and 7th days post challenge to determine the virus shedding using (RRT-PCR).

The collected eggs were then subjected to external and internal egg quality measurements to determine egg quality as follow: group one at the time of collection and then each of the other 4 groups were divided into 2 groups, one of these 2 gropes were stored at a refrigerator at 4 C⁰ and the others group was stored under room temperature. Quality measurement was carried out and recorded at day one and then at the end of week one, two, three and four for respective groups. Parameters tested were egg weight and shell strength as external quality factors, albumin height and Haugh unit as internal quality factors.

Table 2: Challenged vaccinated groups and unvaccinated control groups

| Group No | No of birds | Vaccination at 3 weeks old | Challenged at 6 weeks old |
|----------|-------------|----------------------------------|---|
| Group 1 | 25 | bivalent inactivated IBV vaccine | IBVclassical strain (KP279995) |
| Group 2 | 25 | bivalent inactivated IBV vaccine | IBVvariant2 strain (KP279998) |
| Group 3 | 25 | bivalent inactivated IBV vaccine | Two IBV classical and variant2 strains. |
| Group 4 | 25 | unvaccinated control (+) | classical strain (KP279995) |
| Group 5 | 25 | unvaccinated control (+) | Variant2 strain (KP279998) |
| Group 6 | 25 | unvaccinated control (+) | two IBV classical and variant strains |
| Group 7 | 10 | unvaccinated control (-) | unvaccinated unchallenged |

RESULTS

1. Results of Propagation and titration of IBV strains separately from each other on specific pathogen free embryonated chicken eggs (SPF-ECEs):

Local isolate IBV strains (Chicken / Egypt KP279995 / VACSERA / 2014) (Classical) and (Chicken / Egypt KP279998 / VACSERA / 2014) (variant) were propagated in 9-10 day old, SPF-ECE for 12 passages , allantoic fluids were harvested and tested for sterility. The virus was titrated using infectivity titration on SPF eggs, the virus titer was calculated as titer $10^{8.5}$ EID₅₀/ml. This virus titer was used as the seed for vaccine preparation.

2. Results of Inactivation of IBV strains separately from each other by 0.1% formalin with ensured completion of inactivation

The harvested allantoic fluid of SPF-ECEs after propagation and titration of the viruses are inactivated by using 0.1% formalin solution. IBV was proved to be completely inactivated as indicated by absence of any pathological lesions, and/or deaths of inoculated embryos being inoculated in 9 days old, SPF-ECEs through the allantoic sac and candled daily for 6 days.

3. Quality control tests of the prepared bivalent inactivated IBV vaccine:

3.1. Results of sterility test:

It was found that the prepared bivalent IB vaccine was sterile as it is free from any bacterial and fungal contaminants

3.2. Results of safety test

For safety examination of the prepared bivalent IB vaccine gave neither local nor systemic reactions and no mortalities were observed among inoculated chicks indicating safety of the prepared vaccine.

3.3. Results of potency test

Monitoring of serum antibody titer in chicks vaccinated with inactivated bivalent IBV vaccine using ELISA test:

In order to estimate the antibody titers in chicken groups vaccinated with inactivated bivalent IB vaccine, ELISA test was used. The minimum positive level serum is equal or more than 833 in ELISA antibody titers for inactivated vaccine according to kit manufacture. The mean antibody titers for vaccinated groups estimated all over 3 weeks revealed that it was noticed that chicks vaccinated with inactivated bivalent IB vaccine showed significant increase at 2nd week post vaccination (WPV) (1152.7 ± 1.76) and increased to reached (2789.7 ± 58.4) at the 3rd WPV in comparison with control (SPF chicks non-vaccinated kept as negative control) as shown in table (3) and figure (2).

Table 3: Mean antibody titers after IBV vaccination in vaccinated and control chicks using ELISA test:

| Group/ weeks | Vaccinated groups | Control groups |
|-------------------------|-------------------|----------------|
| Before vaccination | 12.73±7.83 | 11.06±2.31 |
| 1 week post vaccination | 577.3±1.46 | 19.40±9.07 |
| 2 week post vaccination | 1152.7±1.76 | 30.66±2.96 |
| 3 week post vaccination | 2789.7±5.84 | 38.00±3.05 |

Fig. 2: GMT of IBV ELISA reading in both vaccinated and unvaccinated control groups before and after vaccination

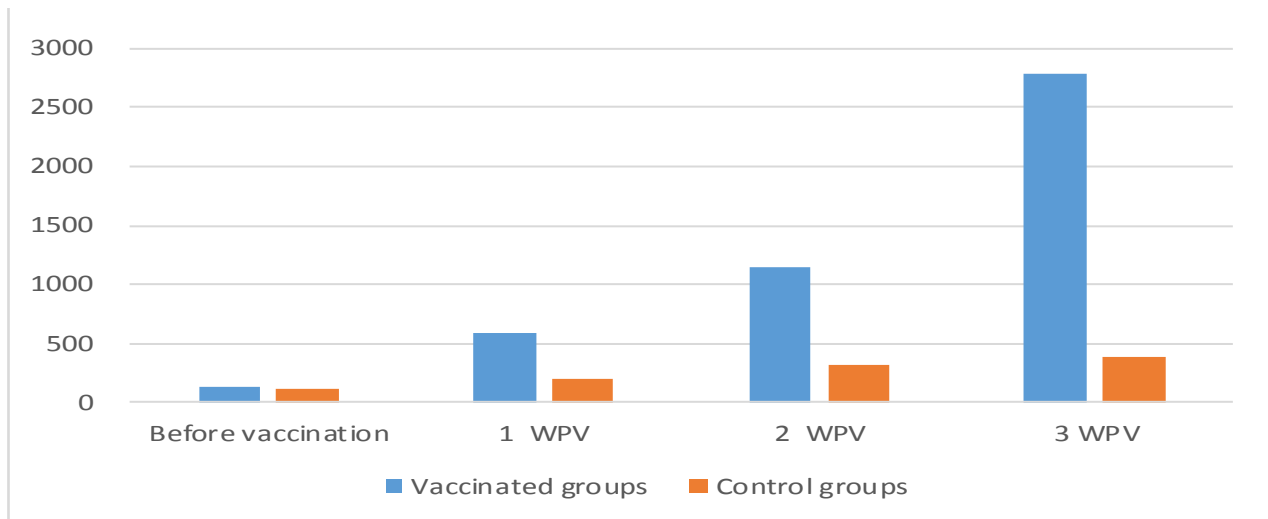


Table 4: Clinical signs score system of infected chicks:

| Clinical signs | Score |
|---|-------|
| No clinical signs | 0 |
| Lacrimation, slight head shaking and watery faces | 1 |
| Lacrimation ,presence of nasal exudates, depression and watery faces | 2 |
| Strong (lacrimation ,presence of nasal exudates, depression) and severe watery faces | 3 |

Scoring index according to Wang and Huang, (2000)

Table 5: Recording clinical signs rate after challenge with challenge strains in vaccinated and unvaccinated control groups:

| Group | Days post challenge and number of dead | | | | | | | | | | Total NO | Total % | Protection % |
|-------|--|-------|---|---|---|---|---|---|---|-------|-------------|------------|-----------------|
| | No | birds | | | | | | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10-15 | | | |
| G1 | - | | 1 | - | - | - | - | - | - | - | 1/25 | 4 | 96 |
| G2 | - | | 1 | - | 1 | - | - | - | - | - | 2/25 | 8 | 92 |
| G3 | - | - | - | 1 | - | - | 1 | - | - | - | 2/25 | 8 | 92 |
| G4 | - | | 2 | - | 5 | - | - | - | 1 | - | 8/25 | 32 | 68 |
| G5 | - | | 4 | 2 | 1 | 3 | 1 | - | 1 | - | 12/25 | 48 | 52 |
| G6 | - | | 5 | 3 | 2 | 1 | 1 | 1 | 1 | 1 | 15/25 | 60 | 40 |
| G7 | - | - | - | - | - | - | - | - | - | - | 0/10 | 0 | 100 |

N.B: Birds in groups (G1, G2, G3) have clinical signs score (1), however in group (G4, G5, G6) have clinical signs score (3).

4. Results of IB vaccine efficacy

Results of challenge test in vaccinated groups with locally prepared vaccine showed that the protection percent against homologous IBV challenge strains was from 96% to 92% with comparison to the control group (40% protection) base on clinical signs, post-mortem examination and viral shedding titers from collected tracheal and kidneys samples at 3rd, 5th and 7th day post challenge from vaccinated and control groups.

Parameters for challenge experiment

Clinical signs rate

All chicks were examined daily for clinical signs of IBV infection such as coughing, sneezing, nasal discharge, head shaking, lethargy and conjunctivitis. The clinical signs were recorded according to scoring index as in table (4).

Post-mortem examination

After challenge, the groups of chicks were be examined for recording of post mortem changes either after death or scarification at interval 3th, 5th and 7th days post infection. However, challenge strains induced depression, ruffled feathers, and respiratory rates; there was low mortality percent was recorded in any of the vaccinated challenged birds in comparison to challenged control one. Upon necropsy, congestion and mucoid inflammation was observed in the trachea in all control positive groups and nephropathogenic variant challenge strain induced kidney swelling, congestion and urate deposition. No gross lesions in both trachea and kidneys were observed in vaccinated

groups compared to control positive birds as showed in figure (3)

Detection of viral shedding after challenge in both trachea and kidney samples

The prepared inactivated vaccine was also able to reduce the shedding titer of the challenge strains at 3rd DPI and 5th DPI in vaccinated birds in comparison to control which were active shedders. By 7th DPI, no virus shedding was detected in all vaccinated groups which it indicated the protection against homologous challenge strains (table 5) and figure (4).

Fig. 3: Post mortem examination in vaccinated and unvaccinated control (+) chicks after challenge.

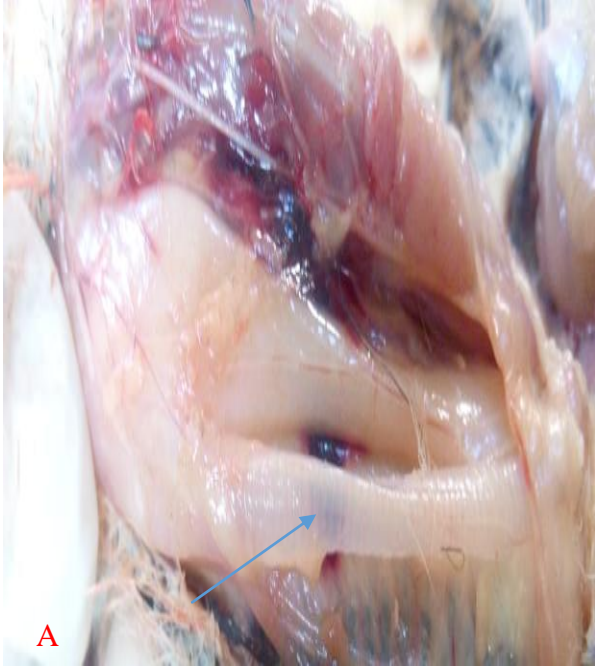

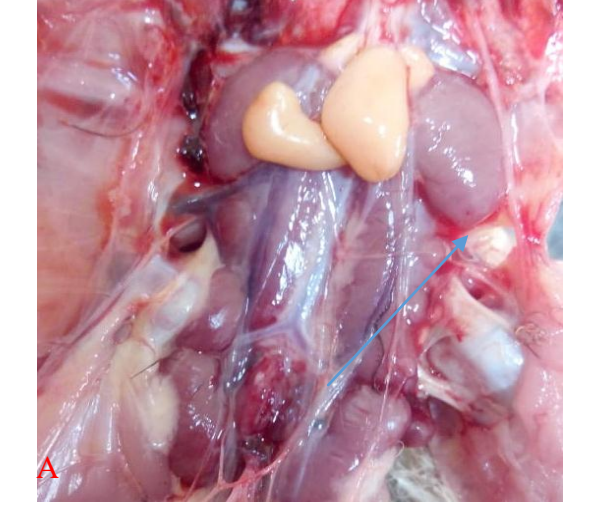

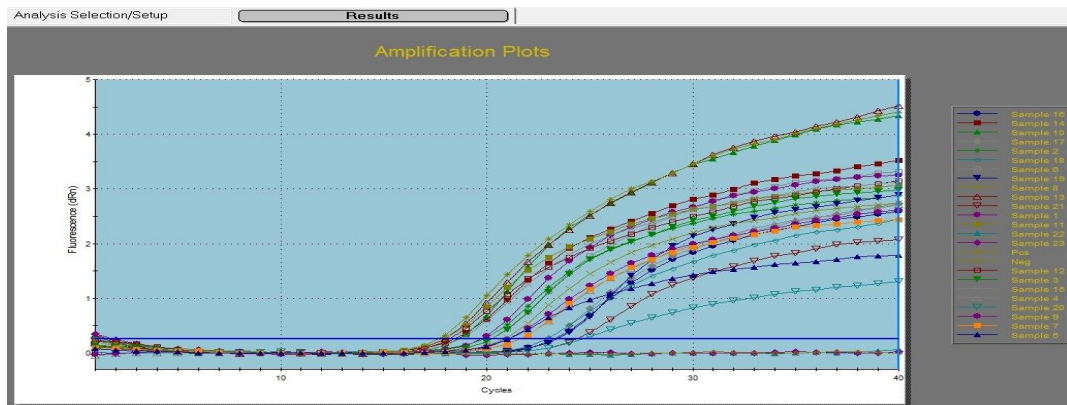
| | |
|--|--|
|  <p>A</p> |  <p>B</p> |
| <p>A: Trachea of vaccinated challenged groups after 5 days from challenge showed normal anatomical structure with transparent appearance</p> | <p>B: Trachea of unvaccinated challenged groups after 5 days from challenge showed mucoid tracheitis</p> |
|  <p>A</p> |  <p>B</p> |
| <p>A: Kidney of vaccinated challenged groups after 7 days from challenge showed normal anatomical structure.</p> | <p>B: Kidney of unvaccinated challenged groups after 7 days from challenge showed severe congestion of renal tubules and urates deposits</p> |

Table 6: The results of RRT-PCR of viral shedding from vaccinated and unvaccinated challenged birds.

| Day post challenge | Group | Result | Cycle threshold (Ct) | Titer (EID50/ml) |
|--------------------|---------|----------|----------------------|---------------------|
| 3 days | Group 1 | Positive | 22.98 | 1.878×10^2 |
| | Group 2 | Positive | 22.87 | 2.028×10^2 |
| | Group 3 | Positive | 20.98 | 2.602×10^2 |
| | Group 4 | Positive | 19.43 | 2.247×10^4 |
| | Group 5 | Positive | 19.85 | 5.675×10^4 |
| | Group 6 | Positive | 20.10 | 3.406×10^4 |
| | Group 7 | Negative | No Ct | (-) |
| 5 days | Group 1 | Positive | 21.08 | 1.089×10^2 |
| | Group 2 | Positive | 21.75 | 1.438×10^2 |
| | Group 3 | Positive | 24.11 | 2.525×10^2 |
| | Group 4 | Positive | 18.75 | 3.614×10^4 |
| | Group 5 | Positive | 18.21 | 5.271×10^4 |
| | Group 6 | Positive | 18.34 | 4.813×10^4 |
| | Group 7 | Negative | No Ct | (-) |
| 7 days | Group 1 | Negative | No Ct | (-) |
| | Group 2 | Negative | No Ct | (-) |
| | Group 3 | Negative | No Ct | (-) |
| | Group 4 | Positive | 18.20 | 5.308×10^3 |
| | Group 5 | Positive | 18.02 | 3.020×10^3 |
| | Group 6 | Positive | 18.76 | 6.589×10^3 |
| | Group 7 | Negative | No Ct | (-) |

Fig. 4: Results of Real-time PCR for viral detection in vaccinated and unvaccinated groups after challenge



DISCUSSION

The widespread exposure to several serotypes and genotypes of IBV with limited cross-protection has led to the failure of currently available vaccines to protect chickens from the heterologous challenge Gelb *et al.* (1991). Many IBV outbreaks associated with respiratory distress, nephropathy, and high mortalities which were mainly attributed to the nephropathogenic IBV variant 2 strains (**Hassan *et al.* . 2016; Zanaty *et al.* 2016; Abozeid *et al.* 2017**).

With the aim to broaden the protective effectiveness of IBV vaccines, an inconsistently successful simultaneous vaccination of imported variant IBV vaccines has been attempted in Egypt .However, the efficacy still does not meet requirements, especially with the significant genetic and antigenic differences between the vaccine and field strains **Hassan *et al.*(2016)**.The use of genetically related strains as vaccines depended upon previous studies that confirmed successful protection using homologous IBV vaccine strain for development local attenuated IBV vaccine from variant strains like Chinese QX-like IBV (**Zhao *et al.*2015; Huo *et al.*2016**), Korean nephropathogenic IBV strains **Lee *et al.* . (2010) and Egyptian attenuated classical and variant 2 IBV Ahmed *et al.* (2018)**.

In Egypt, inactivated vaccines are of one type, Mass (M41), however bivalent vaccines that incorporate additional variant antigens may also be necessary (**Jansen *et al.*,. 2006**).Variant strains may be used to prepare inactivated autogenous vaccines for controlling IB in layers and breeders. Furthermore, the use of vaccines at the manufacturer's recommended dosages will also help avoid back-passage reversion that may be caused by passage in live attenuated vaccines (**OIE,2018**).

The present study was an attempt for preparation of bivalent inactivated oil emulsion IBV vaccine from locally isolated IBV strains, containing both variant strain like IS/1494 (KP279998/2014) and classical strain like H120 (KP279995/2014), this combination for covering the antigenic spectrum of isolates in particular region and it was able to providing sufficient protection against the prevailing field strains. In addition to evaluation of quality control parameters of the locally prepared vaccine to be fit for use in Egyptian poultry farms.

Preparation of bivalent inactivated IBV vaccine carried out through propagation of IBV strains on SPF eggs (9-10) days for 12 passages and allantoic fluids were harvested after 72 hrs post inoculation as described in (**Clarke *et al.* 1972; CFR, 2017; OIE, 2018**).

After collection of infective allantoic fluid of each strain as separate, the virus was titrated using infectivity titration on SPF eggs and titration steps were judged according to the parameters of **CFR, (2017)** in which IBV titer must be not less than $10^{3.5}$ EID₅₀ / dose. So the prepared master seed of IBV strains were satisfactory with $10^{8.5}$ EID₅₀/ml according to Reed and Meunch, (1938) and this viral titer was used as the seed for vaccine preparation. Our result similarly to **Moustafa,(2018)** who formulated inactivated oil emulsion multivalent H5N1, H9N2 and IBV and calculated its infectivity titer for IBV in SPF-ECE to be $10^{8.2}$ EID₅₀/ml.

The harvested allantoic fluids of SPF-ECEs after propagation and titration of IBV strains are inactivated separately in each other by using formalin solution at a final concentration of (0.01%) that comes in accordance with **Beard, (1989)**. Also our results come in accordance with (**Jackwood *et al*, 1997; OIE, 2018**) who proved that IBV was to be completely inactivated as indicated by absence of any pathological lesions, and/or deaths of inoculated embryos being inoculated in 9 days old, SPF-ECEs after 3 successive passage and the batches of inactivated virus suspension must be tested for residual infectivity using embryonated eggs.

The inactivated IBV strains (variant strain KP279998/2014 and classical KP279995/2014) were used as the seed virus for vaccine preparation as recommended by **OIE,(2018)**. Vaccine was prepared in the formula of water-in oil (W/O) emulsion according to **El-Sayed; (2014)** using Montanide™ ISA71 RVG adjuvant was prepared according to the instructions of the manufacturing company of **SEPPIC;(2012)**.

The immunopotentiating effect of W/O inactivated vaccine discussed by **Degen *et al.* (2003)** that B-cell priming requires recognition of free antigen fragments by surface-bound immunoglobulin receptors, it is remarkable that the W/O type of emulsion delayed release features of a vaccine formulation and sustained presence of antigen which improved humoral immune responses.

For evaluation of quality control, we tested sterility, safety and potency of the prepared vaccine. Our results revealed that sterility test of master seed vaccine are free from aerobic and anaerobic bacterial and fungal contamination. That observations agree with parameters of (**European pharmacopoeia, 2013 and 2017**) in which the master seed must be sterile and free from any contamination. Introduction of a reference to requirements for healthy chicken flocks for production

of inactivated vaccines should provide guarantees with regard to extraneous agents contamination.

For safety protocol of vaccine preparation, we inject one group of SPF chicks at three weeks old with double field dose vaccine and kept the other group as unvaccinated control which was conducted according to the requirement of **OIE, (2018)**. After two weeks of inoculation, the SPF chicks gave no abnormal local or systemic serious signs or deaths during the observation period (21 days post inoculation) and consider being safe. Our results came in agreement with the **European pharmacopoeia, (2013)**.

For studying potency parameter of the prepared vaccine, we applied experimental design in SPF chicks and monitoring antibody response post vaccination in the collected sera of vaccinated chicks and compared with control unvaccinated group by ELISA test. Our experimental study came in accordance with **OIE, (2018)** which stated that the potency test is developed from the results of efficacy tests on the furthest passage from the master seed virus and potency of inactivated vaccines evaluated by measuring antibody production through vaccinating 10 SPF chickens for at least 2 weeks of age. The estimation of mean antibody titer recorded 577.3 ± 14.6 , 1152.7 ± 1.76 and 2789.7 ± 58.4 in 1st, 2nd and 3rd week post vaccination in comparison with unvaccinated negative control.

Our results come in accordance with **Mockett and Darbyshire, (1981)** who demonstrated that indirect ELISA was used to detect IgG class-specific antibodies to IBV and that technique was comparatively a very sensitive test, especially when drawing the base line as stated, and also was more sensitive than neutralization or haemagglutination-inhibition methods of antibody assay. ELISA is most suitable for routine serological testing and can detect antibodies caused by vaccination and field exposure. ELISA can detect antibody responses to all serotypes that come with agreement of **OIE, (2018)**.

After three weeks from vaccination, we carried out homologous challenge test with intraocular of $10^{4.5}$ EID₅₀ / ml IBV strains classical / variant either separated or combined in SPF chicks that were divided into six groups as shown in table (2). Similar protocol design according to **OIE, (2018)** which reported that to assess an inactivated vaccine intended to protect birds against respiratory disease. Three parameters were used for assessment of vaccine potency effect, clinical signs, post-mortem examination and detection of viral shedding rate in protective birds and compared with

control group by (RRT-PCR). Our results revealed that the protection % for vaccinated birds gave higher protection rate with vaccinated challenged groups (92%) with comparison to control (+ ve) groups either in 3th, 5th and 7th days post challenge.

A related study carried out by **Ahmed et al. (2018)** who reported that the efficacy of attenuated vaccines showed 90 to 100% protection against the homologous challenge based on ciliostasis score and protection percent. Our results come in accordance to **OIE, (2018)** protocol in which reported that protection % should be at least 80% of the unvaccinated controls should display complete ciliostasis, while tracheal swabs from vaccinated birds should be at least 90% negative for virus isolation.

This results were agree with (**Callison, 2006; CFR, 2017; Egyptian pharmacopoeia, 2017; OIE, 2018**) who reported that the real-time RT-PCR test used to rapidly distinguish IBV from other respiratory pathogens, which is important for control of this highly infectious virus and also that test was extremely sensitive and specific, and can be used to quantitate viral genomic RNA in clinical samples. Similarly, **Roh et al. (2014)** who used Real-time (RRT-PCR) assays to detect the presence of challenge virus when the efficacy of infectious bronchitis virus (IBV) vaccine against field viruses is being experimentally evaluated.

CONCLUSION

These findings provide evidence of the efficacy of the inactivated IBV strains (classical H120 and variant2 IS/1494/06) as vaccine with 96 % of homologous protection. Further studies are required to evaluate the field safety and effectiveness of inactivated viruses, compared to other IBV vaccines. Also, further research should be done to investigate the use of combinations of these inactivated IBV strains to induce broad protection against heterologous IBV challenge.

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