Immunogenicity and Efficacy of a Bivalent Vaccine Against Infectious Bronchitis Virus

Mohammad A. Abdel-Sabour\textsuperscript{1*}, Mohammed A Rohaim\textsuperscript{2*}, Owais J.A. Salman\textsuperscript{1}, Samah E. Abodalal\textsuperscript{1}, Faten F. Mohammad\textsuperscript{3}, Mohammad S. Madkour\textsuperscript{1}, Nabil A. Abdel-Wanis\textsuperscript{1},

Muhammad Munir\textsuperscript{4#}

\textsuperscript{1}Department of Poultry Viral Vaccines, Veterinary Serum and Vaccine Research Institute (VSVRI), Agriculture Research Centre (ARC), Cairo 11381, Egypt

\textsuperscript{2}Department of Virology, Faculty of Veterinary Medicine, Cairo University, Giza 12211, Egypt

\textsuperscript{3}Department of Pathology, Faculty of Veterinary Medicine, Cairo University, Giza 12211, Egypt.

\textsuperscript{4}Division of Biomedical and Life Sciences, Faculty of Health and Medicine, Lancaster University, Lancaster LA1 4YG, UK

\#Corresponding Author: Muhammad Munir, muhammad.munir@lancaster.ac.uk;

+441524595083

* Contributed equally
Abstract

Infectious bronchitis (IB) is a highly contagious viral disease and is responsible for considerable economic losses in the poultry industry, worldwide. To mitigate the IB-associated losses, multiple vaccines are being applied in the sector with variable successes and thus necessitating the development of a potent vaccine to protect against the IB in the poultry. In the present study, we investigated a bivalent live attenuated vaccine consisting of IB virus (IBV) strain H120 (GI-1 lineage) and D274 (GI-12 lineage) to evaluate its protection against heterologous variant of IBV (GI-23 lineage) in chicken. Protection efficacy was evaluated based on the serology, clinical signs, survival rates, tracheal and kidney histopathology and the viral shedding. Results demonstrated that administering live H120 and D274 (named here Classivar®) vaccine in one day-old and 14 days-old provided 100% protection. We observed a significant increase in the mean antibody titers, reduced virus shedding, and ameliorated histopathology lesions compared to routinely used vaccination regimes. These results revealed that usage of different IBV vaccines combination can successfully ameliorate the clinical outcome and pathology in vaccinated chicks especially after booster vaccination regime using Classivar®. In conclusions, our data indicate that Classivar® vaccine is safe in chicks and may serve as an effective vaccine against the threat posed by commonly circulating IBV strains in the poultry industry.

Key words: Infectious bronchitis, Egypt, Protection, GI-23 lineage, Chickens
1. Introduction

Infectious bronchitis (IB) is a highly contagious viral respiratory disease of poultry caused by infectious bronchitis virus (IBV) [1, 2]. The IBV strains can potentially mutate and lead to continuous emergence of a number of serotypes or genotypes, worldwide [3]. Because of adaptive evolution, genetic diversity of coronaviruses is mediated through recombination events and mutations such as substitutions, deletion, and insertion within the viral genome. The low proofreading capacity of RNA-dependent RNA polymerase is attributed to the high mutation rates, whereas recombination results from a unique template copy-choice mechanism during the RNA replication [1, 2, 4, 5]. Continuous emergence of variant IBV strains is often responsible for devastating IB outbreaks in even vaccinated chicken flocks [1, 2, 4, 5].

Previous studies in Egypt have shown that multiple IBV variants were circulating in the Egyptian poultry flocks. Emergence of new IBV variants with nephropathogenic properties is characteristic of the recent outbreaks in Egypt during the last decade causing great economic losses in the poultry industry [6-16]. The GI-23 lineage of IBVs has been reported throughout the Middle East and North Africa [17], Iraq [18], Turkey [19], Libya [20] and Egypt [6-16]. The high incidence of IBV outbreaks in vaccinated poultry flocks is attributed to the circulation of Egyptian variant-2 viruses, which display high genetic differences compared to all imported IBV vaccines and have multiple amino acid substitutions at antigenic epitopes [21, 22]. In field conditions, chickens are simultaneously exposed to different IBV variant strains. Therefore, new vaccines cannot be produced against every evolving strain [23, 24]. However, new vaccines such as IBV strain 793B [25], IB strain QX-like vaccines [26] or IB-VAR2 [27, 28] were used to improve the protective levels. Previous studies have revealed that cross-protection between different IBV strains could range from very poor to moderate. Therefore, it is imperative to assess the cross-protection of vaccine combinations against IBV strains of different serotypes as an alternative approach to control IB in the poultry industry.

Compared to vaccination with one serotype, the usage of different combinations of live IBV vaccines can induce stronger and wider protection against heterologous variant IBV strains [29-31]. Terregino et al. [30] have reported simultaneous or alternate usage of Ma5 and 793B strains, which has induced high levels of protection against heterologous IBV types such as D1466 or QX strains. This protection might be attributed to increased cellular and local immune responses [22, 24]. In order to identify additional combination for better vaccination, this study was designed.
to evaluate the protection conferred by a bivalent live attenuated IBV vaccine (H120 classical strain and D274 variant strain) against challenge with heterologous Egyptian variant IBV (GI-23 lineage). The applied vaccine approached offered full protection, significant increase in the mean antibody titers, reduced virus shedding, and ameliorated histopathology compared to routinely used vaccination regimes.

2. Material and methods

2.1. Viruses and Animals

The IBV classic strain H120 (GI-1 lineage) and variant D274 strain (GI-12 lineage) were kindly provided by the Animal and Plant Health Agency, UK and University of Arkansas, USA; respectively. Both viruses were propagated in 9-day old specific pathogen free embryonated chicken eggs (SPF-ECEs). The allantoic fluid was harvested, dispensed into vials, lyophilized and stored at -80°C as master seed. The Classivar® is a registered trade name at the General Organization for Veterinary Services (GOVS), developed by the authors of the current study for bivalent live attenuated infectious bronchitis vaccine (H120 and D274).

Variant infectious bronchitis virus strain VSVRI_G9 (KP729422.1) was used as a challenge virus which had been previously isolated, characterized and classified as GI-23 lineage which is the commonly circulating lineage in the Egyptian poultry sectors in last five years [8-16]. Experimental SPF chicks were obtained from Nile-SPF-Eggs Farm, Koom Oshiem, Fayoum, Egypt. All chicks were maintained in isolators under positive pressure in air-conditioned rooms, and food and water were provided ad libitum. International, national, and/or institutional guidelines for the care and use of chicks involved in this research was carried out in accordance with Veterinary Serum and Vaccine Research Institute (VSVRI) (e.g., Guidelines for the Veterinary Care of Laboratory Animals). The animal use protocol was reviewed and approved by the Ethics Committee of the Veterinary Serum and Vaccine Research Institute (VSVRI), ARC, Egypt.

2.2. Vaccines Preparation, Sterility and Safety Testing

Preparation and propagation for the attenuated seed viruses of IBV H120, D274 and bivalent strains (H120 and D274; Classivar®) was carried out in SPF-ECEs according to standard procedures [27, 28]. The harvested allantoic fluids were harvested and titrated in 9 days old SPF-
ECEs according to Reed and Muench method [34]. Mixing and dispensing were conducted after obtaining the desired IBV titer for both strains (10^{6.5} EID_{50}/ml) and using skimmed milk as a stabilizer. The three prepared vaccines were tested for sterility, following the standard procedures through cultivation in aerobic and anaerobic bacterial and fungal growth media and was examined daily for a week in terms of any microbial growth [32, 33].

Forty-day-old SPF chicks were randomly divided into four groups (10 chicks/group); group I was inoculated with H120 vaccine, group II was inoculated with D274 vaccine and chicks in the group III were inoculated with bivalent H120 and D274 vaccine (Classivar®) while the group IV left as negative control received the diluent (distal water) for the vaccine. All inoculated chicks received a total of 200 μl allantoic fluid containing 10^{3.5} EID_{50} of each strain as described earlier via oculo-nasal route. All chicks were monitored daily for clinical signs related to IB infection for 14 days post inoculation (dpi), including coughing, sneezing, and tracheal rales.

2.3. Evaluation of Vaccines Potency, Efficacy and Challenge Experiments

A total of 400 one day old SPF chicks were divided into eight equal groups (I-VIII) (50 chicks/group); group I and II and III were vaccinated via oculo-nasal route with Classivar®, D274 and H120 at one day old, respectively (Fig. 1). Group IV was vaccinated via oculo-nasal route with Classivar® at one day old then received a booster dose at 14 days old (Fig. 1). Group V was vaccinated via oculo-nasal route with D274 vaccine at one day old then received a booster dose at 14 days old by the same route (Fig. 1). Group VI was vaccinated via oculo-nasal route with H120 vaccine at one day old then received a booster dose at 14 days old. While group VII was kept as non-vaccinated non-challenged control (negative control) received the diluent for the vaccine and group VIII was kept as non-vaccinated challenged (positive control) (Fig. 1). Each chick within the vaccinated groups was given a dose of 10^{3.5} EID_{50} of each vaccine. Sera samples were collected weekly from each group for up to six weeks post-vaccination for serological investigation using ELISA [32, 33]. Evaluation of the vaccines-induced immune responses were conducted based on ELISA kit (IDEXX) according to manufacturer instructions. All vaccinated chicks were challenged on day 21 post first vaccination through oculonasal route using local Egyptian reference strain IBV variant VSVRI_G9 and the challenge dose 100ul containing 10^{4} EID_{50} that is titrated by Reed and Muench method [34]. The challenged chicks were kept under daily observation for two weeks post challenge with recording of clinical signs, postmortum lesions especially for trachea and kidney,
trachea swabs and sera samples were collected day after post challenge. The challenged chicks 
were humanly killed at the 5th and 7th day post challenge. Five chicks from all challenged groups 
were euthanized with collection of trachea and kidney to determine virus shedding, histopathology 
examination and tracheal and kidney scoring [32, 33].

2.4. Virus Shedding and Histopathology

Virus isolation from tracheal swabs and kidney were carried out through inoculation of 
tracheal swabs and kidney homogenates into 9 days old SPF-ECEs via the chorio-allantoic sac 
according to the standard procedures [32, 33]. Seven days post-inoculation, embryos were 
evaluated for IBV lesions such as stunting, curling, and kidney urates. Likewise, calculation of 
number of chicks protected against IBV and also calculation of the relative protection which is 
represented by the percentage of eggs inoculated in which no IBV lesions was detected [32, 33]. 
Tracheal and kidney specimens were collected from dead/euthanized chicks of different challenged 
groups after challenge, and fixed in 10% buffered neutral formalin for 48 h at room temperature. 
These samples were embedded in paraffin wax and cut into 5-mm sections. The sections were 
stained with Hematoxylin and Eosin stain, and examined for lesions using light microscope [35]. 
Likewise, scoring of tracheal and renal lesions were carried out [36, 37].

2.5. Statistics

The differences in antibody titers and virus shedding titers were estimated using one-way 
ANOVA with Tukey’s post-test through GraphPad Prism version 6 (GraphPad Software, San 

3. Results

3.1. Evaluation of Vaccine Safety and Immune Responses

Three vaccinated groups were observed for 14 days post-vaccination and no clinical, local, 
systemic and necropsy lesions were noticed compared to birds in negative control group. On day 
14 (day of booster vaccination), chicks of group I, II and III exhibited significantly higher antibody 
 titre (p<0.05) compared to non-vaccinated negative control group (Fig. 2a). On the other hand, on 
day 21 of age (day of challenge), vaccinated groups that received booster dose groups IV, V and 
VI) showed significantly (P value <0.0001) higher levels of antibody titres compared to group I,
II and III (single dose vaccination). While chicks of group IV exhibited significantly ($P$ value $<0.0001$) higher antibody titre compared to groups V and VI (Fig. 2a), the antibody titres in groups V and VI were non-significantly different.

3.2. Vaccines Efficacy against Heterologous IBV Challenge, Clinical signs and Gross lesions

All non-vaccinated and challenged chicks showed typical signs of IBV infection on second day post challenge (dpc) which include depression with ruffled feathers, tracheal rales, sneezing and coughing. In contrast, no clinical signs were recorded in non-vaccinated and non-challenged group (negative control). However, less clinical signs were recorded in groups I, II and III (single dose vaccination regime) compared to group VIII (positive control) that was significantly different. On the other hand, group IV (Classivar® vaccination regime) remained free of clinical signs until the end of the experiment with 100% protection compared to all vaccinated groups (Fig. 2b).

Meanwhile groups V and VI (received booster dose D274 and H120, respectively) were able to ameliorate the clinical outcome of IBV infection and showed significant reduction (less sever) of clinical signs compared to a single dose vaccination regime and positive control group. Three days after challenge, congestion of the trachea and pale swollen kidneys were observed in chicks of group VIII (non-vaccinated and challenged) compared to the non-vaccinated and non-challenged (negative control) group that remained free of any gross lesions (Fig. 2b). These pathognomonic gross lesions were also found in chickens in group I, II and III without significant differences ($P$ value $\geq 0.5$). However, chicks of vaccinated groups IV did not show any gross lesions while groups V and VI showed slight congestion of trachea that was significantly different and were less than single vaccination regimes.

3.3. Virus Shedding and Histopathological lesions

The IBV shedding titers were monitored in all vaccinated challenged groups at 0, 3rd, 5th, 7th, 10th and 14th day post challenge (dpc) compared to positive and negative control groups. There was a significant reduction in the IBV shedding titers starting from 3rd day dpc in groups IV, V and VI (vaccinated challenged groups) (Fig. 2c). However, the significance difference ($P$ value $<0.0001$) was obvious in group IV primed and boosted by Classivar® vaccine. On the other hand, there was a slight reduction in the virus shedding in all single dose vaccination regime compared to positive control (non-vaccinated challenged) group (Fig. 2c).
Histopathological lesion scores in group VIII (non-vaccinated challenged control) showed a highest lesion scores compared to all different vaccination regimes. The histopathological alterations in the tracheal mucosa was characterized by severe tracheatitis, diffuse thickening of tracheal mucosa, extensive deciliation of tracheal epithelium with marked necrosis of epithelium, depletion of goblet cells with multifocal metaplastic changes and diffuse infiltration of lymphocytes with dilatation of blood capillaries (Fig.3). However, kidneys showed diffuse degeneration of tubular epithelium comprising renal cortex extending into medullary with multifocal lymphoplasmocytic aggregation in cortical and medullary interstitium associated with scant interstitial fibroplasia. The inflammatory reaction extending into the ureter that showed vaculoation of mucosal epithelium. While non-challenged chicks (negative control; group VII) revealed normal histological structure of tracheal mucosa and kidney (Fig. 3).

Priming and boosting of chicks with IB-H120 combined with IB-D274 (Classivar®) in group IV showed significantly lower tracheal lesion scores similar to group VI (priming and boosting with IB-H120) while kidneys showed variable lesions especially the renal nephrosis in group VI compared to group IV (Classivar® vaccinated group). Priming and boosting of chicks with IB-D274 (group V) showed moderate tracheitis while the renal lesion scores were significantly ameliorated compared to group VII (positive control) with small focal nephritis and nephrosis (Fig. 3). Severity of the tracheal lesions in different vaccinated challenged groups were significantly reduced compared to the non-vaccinated challenged group (Table 1).

4. Discussion
Despite widespread vaccination with live attenuated or inactivated IBV vaccines worldwide, the Egyptian poultry industry has recently faced an increasing incidence of IB outbreaks [6- 16]. The partial cross-protection and widespread multiple IBV genotypes and serotypes in the poultry industry has led to the failure of currently available vaccines which are either based on classical (Mass 41 and H120) and/or variant (D278, CR88 or 4/91) strains [9, 38-41]. Recent studies have also indicated that several virulent IBV strains are co-circulating in the Middle East including Egypt [11, 12]. The aim of this study was to investigate the use of combinations of live attenuated IBV strains to induce a wider protection against heterologous IBV challenge. Until now, the best strategy for the development of attenuated IBV vaccine strains is mainly depending on continuous passaging in chicken embryos [42]. Safety of three prepared live
vaccines (H120, D274 and Classivar®) was tested via the ocular route in day-old SPF. The absence of any obvious adverse effect in day-old SPF chickens up to the 14 days post vaccination was further confirmed the safety of the prepared vaccines. Previous studies have reported that Mass-type vaccines are not sufficient to protect birds against heterologous variant IBV strains [5, 29]. Efficacy of the prepared vaccines against heterologous challenge was investigated in day-old SPF chickens. It has been reported that IBV antibody titers were higher after vaccination with a booster dose compared to single dose vaccination [20]. Our results showed that the mean ELISA titer against IBV for three prepared vaccines were high while in group IV was significantly higher compared to groups V and VI after receiving a booster dose. After challenge using heterologous Egyptian variant field strain of IBV, there was no any obvious clinical signs in group IV, vaccinated with Classivar® with 100% protection compared to groups V (vaccinated with H120) and VI (vaccinated with D274) that showed 90% protection.

On the other hand, single dose vaccination regimes revealed a total of 70% protection. However, non-vaccinated challenged chicks (group VIII) showed characteristic clinical features for IBV infection such as depression, ruffled feather, nasal discharge, wet eyes and difficult breathing. In addition, there was no any clear necropsy lesions with the postmortem examination in group IV. Recent studies highlighted the detection of viral shedding for the evaluation of protection levels afforded by the IBV vaccine candidates [27, 28]. Moreover, a significant reduction in the virus shedding titers was observed in the vaccinated birds at 3 dpi and no virus was detected at 5 and 7 dpi. Viral shedding evaluation revealed that all vaccinated groups I, II, III, V and VI were able to reduce the virus shedding with non-significantly different compared to group IV vaccinated group with Classivar® that was able to significantly reduce the virus shedding compared to other vaccinated groups. Our results revealed that usage of different IBV vaccines combination can ameliorate the pathological alterations in the trachea and kidney of vaccinated challenged chicks especially with booster vaccination regime using bivalent H120 and D274 (Classivar®). These findings indicate that a bivalent IBV vaccine can suitably used as vaccine candidate to provide full protection against the challenge with heterologous Middle Eastern GI-23 IBV strains.

5. Conclusions
Our study provides an evidence of the efficacy of a bivalent H120 and D274 (Classivar®) vaccine candidate against Middle Eastern variant GI-23 IBV strains with full protection to reduce the economic losses caused by variant IBV infections. Likewise, our findings indicated that booster vaccination regimes are better than single vaccination regimes that show high antibody titers with significant reduction in virus shedding and histopathological alterations compared to challenged non-vaccinated chicks. However, further studies are required to evaluate the field safety and efficacy of these vaccines compared to other IBV vaccines especially due to circulation of several virulent IBV strains in the Middle East including Egypt.

Declaration of Competing Interest

All authors declare that there is no conflict of interest.

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Figure captions:

Fig. 1. Experimental plan for the assessment of the prepared IBV vaccines regimes and their efficacies in chickens.

Fig. 2. Evaluation of the prepared vaccines immunogenicity and efficacies. (a) Immunogenicity evaluation and antibody titers based on ELISA in response to vaccination with different vaccine regimes compared to non-vaccinated group. (b) Percentage survival rates for the challenged vaccinated groups with different vaccination regimes, non-vaccinated challenged (positive control) compared with non-vaccinated-non challenged (negative control) group. (c) Virus shedding evaluation for the challenged vaccinated groups, challenged non vaccinated group (positive control) compared to non-challenged-non vaccinated (negative control) group based on real time RT-PCR on the collected Oropharyngeal swabs.

Fig. 3. Histopathologic sections of trachea and kidney stained by H&E. Non challenged- non vaccinated chicks (negative control) showing (A) histological normal tracheal epithelium containing cilia; (B) architecture of renal tubular epithelium, interstitium and glomeruli. Challenged-non vaccinated chicks showing (C) severe deciliation, necrosis of tracheal epithelium, disappearance of goblet cells associated with massive lymphocytic infiltration and dilated blood capillaries; (D) vacuolization and necrosis if renal tubular epithelium with pyknotic nuclei. Group IV, challenged vaccinated chicks with booster dose of bivalent Classivar® vaccine showing (E) focal deciliation of tracheal mucosa with increased size of mucous cells indication activation associated with scarce lymphocytes infiltration in lamina propria; (F) small infiltration of lymphocytes in renal interstitium. Group V, challenged vaccinated chicks with booster dose of D274 vaccine showing (G) thickening of tracheal mucosa, deciliation and focal cuboidal metaplasia of tracheal epithelium, disappearance of mucous glands and intense lymphocytes infiltration and congestion of blood vessels; (H) Mild infiltration of lymphocytes in renal interstitium. Group VI, challenged vaccinated chicks with booster dose of H120 vaccine showing; (I) apparently normal tracheal mucosa (J) Multifocal infiltration of lymphocytes in renal interstitium.