1 **Immunogenicity and Efficacy of a Bivalent Vaccine Against Infectious Bronchitis Virus**

2 Mohammad A. Abdel-Sabour[1*], Mohammed A Rohaim[2*], Owais J.A. Salman[1], Samah E.
3 Abodalal[1], Faten F. Mohammad[3], Mohammad S. Madkour[1], Nabil A. Abdel-Wanis[1],
4 Muhammad Munir[4#]

5 [1]Department of Poultry Viral Vaccines, Veterinary Serum and Vaccine Research Institute
6 (VSVRI), Agriculture Research Centre (ARC), Cairo 11381, Egypt
7
8 [2]Department of Virology, Faculty of Veterinary Medicine, Cairo University, Giza 12211, Egypt
9
10 [3]Department of Pathology, Faculty of Veterinary Medicine, Cairo University, Giza 12211, Egypt.
11
12 [4]Division of Biomedical and Life Sciences, Faculty of Health and Medicine, Lancaster University,
13 Lancaster LA1 4YG, UK
14
15
16
17
18 [#]**Corresponding Author:** Muhammad Munir, muhammad.munir@lancaster.ac.uk;
19 +441524595083
20
21 * Contributed equally
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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

89 to evaluate the protection conferred by a bivalent live attenuated IBV vaccine (H120 classical
90 strain and D274 variant strain) against challenge with heterologous Egyptian variant IBV (GI-23
91 lineage). The applied vaccine approached offered full protection, significant increase in the mean
92 antibody titers, reduced virus shedding, and ameliorated histopathology compared to routinely
93 used vaccination regimes.

94

95 2. Material and methods

96 *2.1. Viruses and Animals*

97 The IBV classic strain H120 (GI-1 lineage) and variant D274 strain (GI-12 lineage) were
98 kindly provided by the Animal and Plant Health Agency, UK and University of Arkansas, USA;
99 respectively. Both viruses were propagated in 9-day old specific pathogen free embryonated
100 chicken eggs (SPF-ECEs). The allantoic fluid was harvested, dispensed into vials, lyophilized and
101 stored at -80°C as master seed. The Classivar® is a registered trade name at the General
102 Organization for Veterinary Services (GOVS), developed by the authors of the current study for
103 bivalent live attenuated infectious bronchitis vaccine (H120 and D274).
104 Variant infectious bronchitis virus strain VSVRI_G9 (KP729422.1) was used as a
105 challenge virus which had been previously isolated, characterized and classified as GI-23 lineage
106 which is the commonly circulating lineage in the Egyptian poultry sectors in last five years [8- 16].
107 Experimental SPF chicks were obtained from Nile-SPF-Eggs Farm, Koom Oshiem, Fayoum,
108 Egypt. All chicks were maintained in isolators under positive pressure in air-conditioned rooms,
109 and food and water were provided *ad libitum*. International, national, and/or institutional
110 guidelines for the care and use of chicks involved in this research was carried out in accordance
111 with Veterinary Serum and Vaccine Research Institute (VSVRI) (e.g., Guidelines for the
112 Veterinary Care of Laboratory Animals). The animal use protocol was reviewed and approved by
113 the Ethics Committee of the Veterinary Serum and Vaccine Research Institute (VSVRI), ARC,
114 Egypt

115

116 *2.2. Vaccines Preparation, Sterility and Safety Testing*

117 Preparation and propagation for the attenuated seed viruses of IBV H120, D274 and
118 bivalent strains (H120 and D274; Classivar®) was carried out in SPF-ECEs according to standard
119 procedures [27, 28]. The harvested allantoic fluids were harvested and titrated in 9 days old SPF-

120 ECEs according to Reed and Muench method [34]. Mixing and dispensing were conducted after
121 obtaining the desired IBV titer for both strains ($10^{6.5}$ EID_{50}/ml) and using skimmed milk as a
122 stabilizer. The three prepared vaccines were tested for sterility, following the standard procedures
123 through cultivation in aerobic and anaerobic bacterial and fungal growth media and was examined
124 daily for a week in terms of any microbial growth [32, 33].
125 Forty-day-old SPF chicks were randomly divided into four groups (10 chicks/group);
126 group I was inoculated with H120 vaccine, group II was inoculated with D274 vaccine and chicks
127 in the group III were inoculated with bivalent H120 and D274 vaccine (Classivar®) while the group
128 IV left as negative control received the diluent (distal water) for the vaccine. All inoculated chicks
129 received a total of 200 µl allantoic fluid containing $10^{3.5}$ EID_{50} of each strain as described earlier
130 via oculo-nasal route. All chicks were monitored daily for clinical signs related to IB infection for
131 14 days post inoculation (dpi), including coughing, sneezing, and tracheal rales.
132
133 *2.3. Evaluation of Vaccines Potency, Efficacy and Challenge Experiments*
134 A total of 400 one day old SPF chicks were divided into eight equal groups (I-VIII) (50
135 chicks/group); group I and II and III were vaccinated via oculo-nasal route with Classivar®, D274
136 and H120 at one day old, respectively (Fig. 1). Group IV was vaccinated via oculo-nasal route with
137 Classivar® at one day old then received a booster dose at 14 days old (Fig. 1). Group V was
138 vaccinated via oculo-nasal route with D274 vaccine at one day old then received a booster dose at
139 14 days old by the same route (Fig. 1). Group VI was vaccinated via oculo-nasal route with H120
140 vaccine at one day old then received a booster dose at 14 days old. While group VII was kept as
141 non-vaccinated non-challenged control (negative control) received the diluent for the vaccine and
142 group VIII was kept as non-vaccinated challenged (positive control) (Fig. 1). Each chick within the
143 vaccinated groups was given a dose of $10^{3.5}$ EID_{50} of each vaccine. Sera samples were collected
144 weekly from each group for up to six weeks post-vaccination for serological investigation using
145 ELISA [32, 33]. Evaluation of the vaccines-induced immune responses were conducted based on
146 ELISA kit (IDEXX) according to manufacturer instructions. All vaccinated chicks were challenged
147 on day 21 post first vaccination through oculonasal route using local Egyptian reference strain IBV
148 variant VSVRI_G9 and the challenge dose 100ul containing 10^4 EID_{50} that is titrated by Reed and
149 Menuch method [34]. The challenged chicks were kept under daily observation for two weeks post
150 challenge with recording of clinical signs, postmotum lesions especially for trachea and kidney,

151 trachea swabs and sera samples were collected day after post challenge. The challenged chicks
152 were humanly killed at the 5th and 7th day post challenge. Five chicks from all challenged groups
153 were euthanized with collection of trachea and kidney to determine virus shedding, histopathology
154 examination and tracheal and kidney scoring [32, 33].
155
156 *2.4. Virus Shedding and Histopathology*
157 Virus isolation from tracheal swabs and kidney were carried out through inoculation of
158 tracheal swabs and kidney homogenates into 9 days old SPF-ECEs via the chorio-allantoic sac
159 according to the standard procedures [32, 33]. Seven days post-inoculation, embryos were
160 evaluated for IBV lesions such as stunting, curling, and kidney urates. Likewise, calculation of
161 number of chicks protected against IBV and also calculation of the relative protection which is
162 represented by the percentage of eggs inoculated in which no IBV lesions was detected [32, 33].
163 Tracheal and kidney specimens were collected from dead/euthanized chicks of different challenged
164 groups after challenge, and fixed in 10% buffered neutral formalin for 48 h at room temperature.
165 These samples were embedded in paraffin wax and cut into 5-mm sections. The sections were
166 stained with Hematoxylin and Eosin stain, and examined for lesions using light microscope [35].
167 Likewise, scoring of tracheal and renal lesions were carried out [36, 37].
168
169 *2.5. Statistics*
170 The differences in antibody titers and virus shedding titers were estimated using one-way
171 ANOVA with Tukey's post-test through GraphPad Prism version 6 (GraphPad Software, San
172 Diego California USA, www.graphpad.com).
173
174 **3. Results**
175 *3.1. Evaluation of Vaccine Safety and Immune Responses*
176 Three vaccinated groups were observed for 14 days post-vaccination and no clinical, local,
177 systemic and necropsy lesions were noticed compared to birds in negative control group. On day
178 14 (day of booster vaccination), chicks of group I, II and III exhibited significantly higher antibody
179 titre ($p < 0.05$) compared to non-vaccinated negative control group (Fig. 2a). On the other hand, on
180 day 21 of age (day of challenge), vaccinated groups that received booster dose groups IV, V and
181 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 observed 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 ≥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).

213 Histopathological lesion scores in group VIII (non-vaccinated challenged control) showed
214 a highest lesion scores compared to all different vaccination regimes. The histopathological
215 alterations in the tracheal mucosa was charcterized by severe tracheatitis, diffuse thickening of
216 tracheal mucosa, extensive deciliation of tracheal epithelium with marked necrosis of epithelium,
217 depletion of goblet cells with multifocal metaplastic changes and diffuse infiltration of
218 lymphocytes with dialtation of blood capillaries (Fig.3). However, kidneys showed diffuse
219 degeneration of tubular epithellium comprising renal cortex extending into medullary with
220 multifocal lymphoplasmocytic aggregation in cortical and medullary interstitium associated with
221 scant interstitial fibroplasia. The inflammatory reaction extending into the ureter that showed
222 vaculoation of mucosal epithelium. While non-challenged chicks (negative control; group VII)
223 revealed normal histological structure of tracheal mucosa and kidney (Fig. 3).
224 Priming and boosting of chicks with IB-H120 combined with IB-D274 (Classivar®) in
225 group IV showed significantly lower tracheal lesion scores similar to group VI (priming and
226 boosting with IB-H120) while kidneys showed variable lesions especially the renal nephrosis in
227 group VI compared to group IV (Classivar® vaccinated group). Priming and boosting of chicks
228 with IB-D274 (group V) showed modarate tracheitis while the renal lesion scores were
229 significantly ameliorated compared to group VII (positive control) with small focal nephritis and
230 nephrosis (Fig. 3). Severity of the tracheal lesions in different vaccinated challenged groups were
231 significantly reduced compared to the non-vaccinated challenged group (Table 1).
232
233 ## 4. Discussion
234 Despite widespread vaccination with live attenuated or inactivated IBV vaccines
235 worldwide, the Egyptian poultry industry has recently faced an increasing incidence of IB
236 outbreaks [6- 16]. The partial cross-protection and widespread multiple IBV genotypes and
237 serotypes in the poultry industry has led to the failure of currently available vaccines which are
238 either based on classical (Mass 41 and H120) and/or variant (D278, CR88 or 4/91) strains [9, 38-
239 41]. Recent studies have also indicated that several virulent IBV strains are co-circulating in the
240 Middle East including Egypt [11, 12]. The aim of this study was to investigate the use of
241 combinations of live attenuated IBV strains to induce a wider protection against heterologous IBV
242 challenge. Until now, the best strategy for the development of attenuated IBV vaccine strains is
243 mainly depending on continuous passaging in chicken embryos [42]. Safety of three prepared live

244 vaccines (H120, D274 and Classivar®) was tested via the ocular route in day-old SPF. The absence
245 of any obvious adverse effect in day-old SPF chickens up to the 14 days post vaccination was
246 further confirmed the safety of the prepared vaccines. Previous studies have reported that Mass-
247 type vaccines are not sufficient to protect birds against heterologous variant IBV strains [5, 29].
248 Efficacy of the prepared vaccines against heterologous challenge was investigated in day-old SPF
249 chickens. It has been reported that IBV antibody titers were higher after vaccination with a booster
250 dose compared to single dose vaccination [20]. Our results showed that the mean ELISA titer
251 against IBV for three prepared vaccines were high while in group IV was significantly higher
252 compared to groups V and VI after receiving a booster dose. After challenge using heterologous
253 Egyptian variant field strain of IBV, there was no any obvious clinical signs in group IV,
254 vaccinated with Classivar® with 100% protection compared to groups V (vaccinated with H120)
255 and VI (vaccinated with D274) that showed 90% protection.

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

272

273 **5. Conclusions**

274 Our study provides an evidence of the efficacy of a bivalent H120 and D274 (Classivar®)
275 vaccine candidate against Middle Eastern variant GI-23 IBV strains with full protection to reduce
276 the economic losses caused by variant IBV infections. Likewise, our findings indicated that booster
277 vaccination regimes are better than single vaccination regimes that show high antibody titers with
278 significant reduction in virus shedding and histopathological alterations compared to challenged
279 non-vaccinated chicks. However, further studies are required to evaluate the field safety and
280 efficacy of these vaccines compared to other IBV vaccines especially due to circulation of several
281 virulent IBV strains in the Middle East including Egypt.
282
283 **Declaration of Competing Interest**
284 All authors declare that there is no conflict of interest.
285
286 **Acknowledgements**
287 This work was financed by International Foundation for Science (IFS) (project No. I-3-B-
288 6270-1), The Organization of Islamic Cooperation's Standing Committee on Scientific and
289 Technological Cooperation (COMSTECH). The funding sources had no role in the study design,
290 collection, or analysis of the data, writing of the manuscript, or in the decision to submit the
291 manuscript for publication.
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455 **Figure captions:**

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

458 Fig. 2. Evaluation of the prepared vaccines immunogenicity and efficacies. (a) Immunogenicity
459 evaluation and antibody titers based on ELISA in response to vaccination with different vaccine
460 regimes compared to non-vaccinated group. (b) Percentage survival rates for the challenged
461 vaccinated groups with different vaccination regimes, non-vaccinated challenged (positive control)
462 compared with non-vaccinated-non challenged (negative control) group. (c) Virus shedding
463 evaluation for the challenged vaccinated groups, challenged non vaccinated group (positive
464 control) compared to non-challenged-non vaccinated (negative control) group based on real time
465 RT-PCR on the collected Oropharyngeal swabs
466

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

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