

1 **Mucosal, cellular and humoral immune responses induced by different live infectious**
2 **bronchitis virus vaccination regimes and the protection conferred against infectious**
3 **bronchitis virus Q1 strain**

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29

30 **ABSTRACT**

31 The objectives of present study were to assess the mucosal, cellular and humoral immune
32 responses induced by two different infectious bronchitis virus (IBV) vaccination regimes and
33 their efficacy against challenge by a variant IBV Q1. Day-old broiler chicks were vaccinated
34 with live H120 alone (Group I) or in combination with CR88 (Group II). Both groups were
35 again vaccinated with CR88 at 14 days of age (doa). One group was kept as the control
36 (Group III). A significant increase in lachrymal IgA levels was observed at 4 doa, which then
37 peaked at 14 doa in the vaccinated groups. The IgA levels in group II were significantly
38 higher than group I from 14 doa. Using immunohistochemistry to examine changes in the
39 number of CD4+ and CD8+ cells in the trachea, it was found that overall patterns of CD8+
40 were dominant compared to CD4+ cells in both vaccinated groups. CD8+ were significantly
41 higher in group II compared to group I at 21 and 28 doa. All groups were challenged oculo-
42 nasally with a virulent Q1 strain at 28 doa, and their protection was assessed. Both vaccinated
43 groups gave excellent ciliary protection against Q1, though group II's histopathology lesion
44 scores and viral RNA loads in the trachea and kidney showed greater levels of protection
45 compared to group I. These results suggest that greater protection is achieved from the
46 combined vaccination of H120 and CR88 of day-old chicks, followed by CR88 at 14 doa.

47 **Keywords:** Infectious bronchitis virus, Chicken, Vaccination, Mucosal-humoral-cell
48 mediated immune responses, Protection, Q1-challenge

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53 **INTRODUCTION**

54 The prevention of infectious bronchitis (IB) in chickens is achieved through the use of
55 live and inactivated vaccines, which provide protection against virulent field IB viruses in the
56 event of an exposure. Despite these preventative measures, outbreaks of IB frequently occur
57 in many poultry producing countries (1-3). This is probably due to the emergence of new
58 variants of infectious bronchitis virus (IBV) (1-5). For the successful protection of chickens
59 against infection, it is essential to identify the prevalent genotypes in the region and to
60 determine the cross-protective potential of available vaccines and optimise strategic
61 vaccination programmes.

62 IB was first described in the USA during the 1930s and was identified in the UK in
63 1948. Thereafter, many IBV variants were isolated from Europe, significantly a variant called
64 793B that emerged in the 1990s (6). Later, IBV QX was first identified in China (7) before
65 spreading to Europe (8). Another IBV genotype, Q1, genetically and serologically distinct
66 from the classical IBVs, was also reported in China (9), the Middle East (10) and Europe
67 (11). To contain this strain, an effective vaccination programme is needed. However, very
68 little is known about the cross protection induced by the commercially available vaccines or
69 vaccination regimes against this variant Q1.

70 An effective and long-lasting protection against IBV infection requires the activation
71 of effector, memory cell-mediated and humoral immune responses against the virus (12). A
72 number of studies have reported the systemic and local humoral immune response (HIR) to
73 IBV vaccination (12-14). In chickens, experimentally challenged with IBV, the development
74 of a cell mediated immune response (CMI) has been correlated with effective virus clearance,
75 reduction of clinical signs and resolution of lesions (15, 16). The presence of cytotoxic CD8+
76 T lymphocytes (CTL) represents a good correlation for decreasing infection and corresponds
77 with a reduction in clinical signs, as CTL activity is major histocompatibility complex

78 restricted and these T cells mediate cytolysis (17). It has additionally been shown that the
79 transfer of CTLs obtained from spleen of IBV-infected chickens, was protective to naïve
80 chicks against a subsequent IBV challenge (15, 18). During the course of experimental viral
81 infection, Kotani *et al* (2000) showed that the clearance of the IBV from the tracheal mucosa
82 occurred at an early phase of the infection and CTLs at the tracheal mucosa were proposed to
83 be involved in this clearance (19). To date, there is no information available on the tracheal
84 mucosal leukocytes after vaccination with live IBV vaccines. Nevertheless, Okino *et al*
85 (2013) have quantified the relative expression of the CTLs genes in tracheal samples from
86 vaccinated and further challenged birds. The up regulation of these genes, in the tracheal
87 mucosa of the full-dose vaccinated birds, was significantly increased at 24 hours post
88 infection (hpi), demonstrating the development of a CMI memory response (20). However,
89 these researchers did not directly measure the activity of CMI, such as the cytotoxic
90 mechanism of CTLs.

91 Despite all these reports, the kinetics of, and the relationship between local and
92 systemic HIR and CMI induced by different IBV vaccination regimes, needs to be better
93 understood for protection against emerging IBV strains. Thus, the objective of our study was
94 to measure the local as well as systemic HIR and CMI induced by two different IBV
95 vaccination regimes administered to commercial broiler chicks, and to estimate the
96 protection achieved against a recently isolated virulent Q1 strain.

97

98 **MATERIALS AND METHODS**

99 **Birds**

100 One hundred twenty broiler chicks, aged 1-day-old, were obtained from a commercial
101 hatchery. Birds were allowed *ad libitum* access to feed and drinking water. All procedures
102 were undertaken according to the UK legislation on the use of animals for experiments as

103 permitted under the project license PPL 40/3723, which was approved by the University of
104 Liverpool ethical review committee.

105 **Challenge virus**

106 The virulent Q1 isolate used in this study was kindly provided by Merial Animal Health.
107 PCR confirmed that the allantoic fluid, from eggs used to propagate the virus, was free of
108 Newcastle disease, avian influenza, infectious bursal disease, infectious laryngotracheitis and
109 avian metapneumoviruses. Q1 IBV was also free of bacterial or fungal contaminants. The
110 virus was titrated in the chicken tracheal organ culture (TOC) as described before and
111 expressed in 50% (median) ciliostatic doses (CD₅₀)/ml (21).

112

113 **Vaccine preparation**

114 As recommended by the manufacturer (Merial Animal Health Limited, UK), the vaccines
115 were prepared, by thoroughly mixing one vial of live IBV H120 (Bioral H 120[®]) vaccine with
116 100 ml of sterile water (SW). For combined vaccinations, one vial of each Bioral H 120[®] and
117 live IBV CR88 (GALLIVAC[®] IB88) vaccines were mixed together in 100 ml of SW.
118 Immediately after preparation, the vaccines and SW were kept in a cold box (at 0°C). Each
119 chick received a total of 100 µl of the appropriate vaccine ocularly (50 µl) and nasally (50 µl)
120 or SW. To quantify the virus, titration of live IBV vaccine for H120 and CR88 was
121 performed by using 9-11 days of age (doa) specific pathogen free (SPF) embryonated chicken
122 eggs (ECE) inoculated via the allantoic cavity. The ECE were examined for IBV specific
123 lesions (curling and dwarfing) of the embryos up to five days post inoculation. Viral titres
124 were calculated according to Reed *et al.* (22) and expressed as the Egg infective dose
125 (EID₅₀/ml). The titre of the vaccine viruses used was 3.5 log₁₀ EID₅₀/chick and 4.25 log₁₀
126 EID₅₀/chick for the H120 strain and CR88 strain, respectively.

127 **Experimental design**

128 One hundred and twenty broiler chicks, aged 1-day-old, were divided into three groups
129 (n=40 chicks/group) (Table 1). Chicks in Group I were inoculated oculonasally with 100 μ l
130 of live H120 vaccine alone. In group II, chicks were inoculated oculonasally with 100 μ l of
131 both live H120 and CR88 vaccines simultaneously. Chicks in both groups (I and II) were
132 again inoculated with a live CR88 vaccine at 14 doa. Group III received only 100 μ l of SW
133 oculonasally and was kept as a control. Samples (5 birds/group) of serum, tears and
134 heparinized blood were collected at 0, 4, 7, 14, 21 and 28 doa before sacrificing the birds.
135 The tears and serum samples were stored at -20°C, and blood samples were processed
136 immediately for peripheral blood mononuclear lymphocytes isolation. Five chickens from
137 each group per interval were humanely euthanized for the collection of approximately 1 cm
138 of the upper trachea in OCT to be snap-frozen in liquid nitrogen for immunohistochemistry
139 (IHC). The rest of the trachea was used for tracheal washes. At 28 doa, 10 birds from each
140 group were challenged via ocular-nasal route with the Q1 ($10^{4.0}$ CD₅₀/bird) and observed
141 daily for clinical signs. After 5 days post challenge (dpc), all 10 birds from each group were
142 necropsied and tracheal samples were collected; a portion placed in the RNALater® (Qiagen,
143 Crawley, UK) and stored at -70°C until processing for examination of viral RNA load. The
144 remaining portions were examined by histopathology and cilliosistests. The kidneys from
145 all groups were also taken for histopathology and viral RNA load examination.

146

147 **Sample collection for antibody detection**

148 The potential of the vaccines to induce antibody production was assessed individually
149 by using samples of sera, tears and tracheal washes. Tears were collected using sodium
150 chloride as described before (23), immediately centrifuged at 3000 x g for 3 min before
151 storing the supernatant at -70°C until used. To collect the tracheal washes, the trachea was
152 clamped with two artery forceps at both the ends, and washed with 1 ml PBS using a syringe

153 with 19 gauge needle (24). The collected samples were centrifuged at 3000 x g for 3 min, and
154 the supernatant stored at -70°C until further use.

155 **ELISAs**

156 To detect IBV antibodies, sera samples were tested with a commercial IBV ELISA kit
157 (FlockChek[®], IDEXX Laboratories, Inc, Westbrook, ME, USA), and immunoglobulin A
158 (IgA) in tears and tracheal washes was assayed using commercial IgA chicken ELISA kit
159 (Abcam, Cambridge, UK). Both assays were carried out according to the respective
160 manufacturer's instructions.

161 **Haemagglutination inhibition (HI) test**

162 For the HI test, M41 and 793B HA antigens were obtained from GD Animal Health
163 Service (Deventer, Netherlands). The Q1 HA antigen was prepared in our laboratory as
164 described earlier (25). The HI test was conducted according to standard procedures (OIE),
165 using 4 HA units of antigen per well. The HI titres were read as the reciprocal of the highest
166 dilution showing complete inhibition and the HI geometric mean titres were expressed as
167 reciprocal log₂.

168 **Cellular immune responses**

169 **Analysis of T lymphocyte subsets (CD4+:CD8+) ratio in peripheral blood**

170 To determine the percentage of T-lymphocyte subpopulations, blood was collected
171 from the cephalic vein in heparin tubes (Sigma Aldrich Co., St. Louis, MO, USA) at final
172 concentrations of 10 USP/ml of blood, and further diluted (1:1) with RPMI 1640 medium
173 (Sigma Aldrich Co., St. Louis, MO, USA). The prepared blood samples (1 ml each) were
174 then over layered onto 0.5 ml of Histopaque -1.077 gradient (Sigma Aldrich Co., St. Louis,
175 MO, USA) and centrifuged in 1.5 ml Eppendorf vial at 8000 x g for 90 sec. After
176 centrifugation, the buffy coat formed of mononuclear cells was gently collected, washed
177 twice with a RPMI 1640 medium and adjusted to 1×10^7 cells/ml. The cells were resuspended

178 in 0.5% BSA (Sigma Aldrich Co., St. Louis, MO, USA) in PBS (blocking solution) and
179 incubated at room temperature for 15 min. The sample (100 μ l) was incubated with
180 antibodies against surface domains of CD4 (mouse anti-chicken CD4-FITC clone CT-4;
181 0.5mg/ml; Southern Biotech, Birmingham, AL, USA) and CD8 (mouse anti-chicken CD8a-
182 FITC clone CT-8; 0.5mg/ml; Southern Biotech) receptors of T-lymphocytes (antibody final
183 concentrations as 0.2 μ l/100 μ l of sample) for 30 min in the dark. The stained cells were
184 detected by flow cytometry (BD Accuri[®] C6, BD Bioscience San Jose, CA, USA) to count
185 the T lymphocytes. The unstained cell sample was used as a negative control to adjust the
186 threshold.

187 **Immunohistochemical detection of CD4+, CD8+ and IgA-bearing B-cells in tracheal**
188 **sections**

189 The OCT-embedded tracheal samples were cut into 5 μ m sections, fixed in ice-cold
190 acetone for 10 min, air dried at room temperature and stored at -80°C until staining. Just
191 prior to staining, slides were removed from -80°C and air dried at room temperature for 10
192 min. After endogenous peroxidase inhibition using 0.03% hydrogen peroxide in PBS for 20
193 min, the endogenous biotin or biotin-binding proteins in tissue sections were blocked with
194 blocking serum using VECTASTAIN[®] Elite ABC kit (Vector Laboratories, Burlingame,
195 USA). Following blocking, tissue sections were stained overnight at 4°C in the dark to detect
196 CD4+, CD8+ and IgA+ cells by using mouse monoclonal antibodies to chicken CD4 (clone
197 CT-4; 0.5 mg/ml) and CD8a (clone CT-8; 0.5 mg/ml) at 1:1000, and to chicken IgA (clone A-
198 1; 0.5 mg/ml) at 1:2000. All monoclonal antibodies were procured from Southern Biotech,
199 Birmingham, AL, USA. The staining procedure was performed as described earlier (26). For
200 each sample, the average number of positive cells/400 \times microscopic field was calculated for
201 each cell type (26).

202

203 **Ciliary protection**

204 At 5 dpc, trachea samples were evaluated according to standard procedure for ciliary
205 movement, and the ciliary protection for each group was calculated (27).

206 **Histopathological evaluation**

207 At 5 dpc, kidneys and tracheas from humanely euthanized birds were collected and
208 fixed in 10% formalin. The tissues were embedded in paraffin wax (50-60°C) and sections
209 were cut to 7µm thickness. Tissue sections were stained by haematoxylin and eosin (H&E)
210 for microscopic evaluation, the scores attributed according to histopathological severity and
211 determined by recommendations described previously (28, 29).

212 **Real time RT-PCR (RT-qPCR)**

213 Total RNA extractions from the tracheas and kidneys, collected from the challenged
214 birds, were performed immediately using RNeasy® Mini Kit (Qiagen, Crawley, UK)
215 according to the manufacturer's instructions. Quantification of the viral RNA was done by
216 quantitative real-time RT-PCR (RT-qPCR) using IBV 3'untranslated region (UTR) gene-
217 specific primers and probes as described previously (30). The RT-qPCR was performed
218 according to the manufacturer's instructions using the One-Step RT-PCR kit (Qiagen,
219 Crawley, UK) and 40 ng of total RNA per reaction. Amplification plots were recorded and
220 analyzed, the threshold cycle (Ct) determined with Rotor-Gene® Q thermocycler software
221 (Qiagen, Crawley, UK). The Ct values were converted to log relative equivalent units (REU)
222 of viral RNA, done through generation of a standard curve of five 10-fold dilutions of
223 extracted RNA from infective allantoic fluid of a 10⁶ EID₅₀ dose of M41 as described earlier
224 (31).

225

226 **Statistical analysis**

227 The comparisons of the means of anti-IBV antibody levels; CD4+:CD8+ ratio in peripheral
228 blood; immunohistochemical detection of CD4+, CD8+ and IgA-bearing B-cells in tracheal
229 sections were performed using one-way analysis of variance (ANOVA), followed by the
230 post-hoc LSD multiple comparison test using GraphPad™ Prism version 6.00 software.
231 Kruskal-Wallis test followed by Dunn's test was used for statistical analysis of the non-
232 parametric RT-qPCR and histopathological evaluation data. Differences were considered
233 significant at $P<0.05$.

234

235 **RESULTS**

236 **Systemic humoral immune response**

237 **ELISA**

238 On the day of vaccination, the mean of maternally derived anti-IBV antibody titre was
239 1750 ± 203 . Subsequently, the antibody levels in all three groups declined to below cut-off
240 point (396) by 14 doa. After the booster vaccination with the CR88 at 14 doa, a significant
241 increase in the antibody titres till 28 doa was observed in groups I and II, as shown in Fig. 1.
242 On these time points, the levels of antibodies were not significantly different between the
243 vaccinated groups ($P<0.05$). After 14 doa though, the antibody titres in group III was always
244 less than the cut-off value of 396 in this assay.

245 **HI test**

246 The level of serotype specific antibodies against homologous and heterologous
247 antigens was evaluated by a HI test (Table 2). The HI antibody response against all the
248 antigens used showed no significant difference ($P<0.05$) between the groups from 0 to 7 doa.
249 However, a lower antibody response was obtained in all groups when the antigen used in the
250 HI test was heterologous (Q1) to the viruses used in the vaccination. On 14 doa, the mean HI
251 antibody titre to the M41 was significantly higher ($P<0.05$) in group II than group I and III.

252 Thereafter, at 28 doa, the levels of antibodies to M41 in group I and II were very similar and
253 significantly higher than group III ($P<0.05$). At 21 doa, group II showed significant increase
254 of HI antibody response against CR88, following revaccination with a homologous antigen.
255 A similar increase was observed in group I on the same sample day. Thereafter, at 28 doa, the
256 HI antibody titre to CR88 antigen was overall significantly higher in group II (\log_2 8.2)
257 followed by group I (\log_2 4.4) then group III (\log_2 1.8). At 21 doa, the titres to Q1 in groups I
258 and II were higher compared to group III ($P<0.05$). At 28 doa, the mean HI titre to Q1 was
259 significantly higher ($P<0.05$) in group II than group I with a mean difference of 1.2 \log_2 .

260 **Mucosal humoral immune responses**

261 In both, groups I and II, the level of IgA in tears increased significantly ($P<0.05$)
262 compared to control group III from 4 doa, continuing to rise until and initially peaking on 14
263 doa. In the vaccinated groups, after the second vaccination at day 14, IgA values fell, then
264 increased slightly again through to 28 doa, the day of challenge. The IgA levels in group II
265 were significantly higher ($P<0.05$) than group I from 14 doa until 28 doa, the end of the
266 observation period (Fig. 2a). The level of IgA in tracheal washes in both vaccinated groups
267 was detected from 4 doa, peaking at 7 doa before declining till 28 doa. No significant
268 ($P<0.05$) difference in the level of IgA in tracheal washes induced by the two vaccine groups
269 was observed at any doa (Fig. 2b). IgA levels in tears and tracheal washes of both vaccinated
270 groups were significantly higher than the levels from the unvaccinated control group.

271 **Systemic cell-mediated immune response**

272 **CD4+:CD8+ ratio in peripheral blood**

273 Flow cytometry results showed that at 7 doa, the CD4+:CD8+ ratios were slightly
274 higher in both vaccinated groups compared to that of the non-vaccinated group, though there
275 was no significant difference ($P<0.05$) between the CD4+:CD8+ ratios of the vaccinated and
276 non-vaccinated groups observed up to 14 doa (Fig. 3). After the booster vaccination with the

277 CR88 at 14 doa, the ratio of CD4+:CD8+ on 21 doa showed slight increase in both vaccinated
278 groups, being significantly higher ($P<0.05$) in group I compared to that of group II and III.
279 At 28 doa, the ratio was significantly higher ($P<0.05$) in group II compared to groups I and
280 III.

281 **Mucosal cell-mediated immune responses in the trachea**

282 The kinetics of CD4+, CD8+ and IgA-bearing B lymphocytes in the trachea were
283 studied by IHC (Fig. 4). The number of CD4+ lymphocytes in the trachea increased
284 significantly ($P<0.05$) from 4 doa in both vaccinated groups compared to the control (Fig.
285 5a). The number of CD4+ cells reached its peak at 4 doa in group I and at 7 doa in group II,
286 before gradually decreasing until 14 doa. After the second immunization, CD4+ cells
287 strongly increased in number by 21 doa in comparison to the non-vaccinated controls before
288 declining again. The difference between the vaccinated groups I and II was not statistically
289 significant ($P<0.05$). The CD8+ cells subpopulation in groups I and II started to increase
290 significantly ($P<0.05$) at 4 doa, reaching peak at 7 doa and then declining (Fig. 5b). After
291 revaccination with CR88 at 14 doa, both vaccinated groups showed a strong increase in the
292 number of CD8+ cells. The number of CD8+ cells were significantly higher in group II than
293 group I at 21 and 28 doa ($P<0.05$). Overall, the dynamics of the CD8+ cell subpopulations in
294 both vaccinated groups were more dominant than CD4+ cells. At 7 doa, the IgA-bearing B
295 cells increased in vaccinated groups I and II, peaking at 14 doa and showing significant
296 difference compared with the unvaccinated group ($P<0.05$). The number of IgA-bearing B
297 cells was significantly higher in group II than group I at 21 doa, whereas, no significant
298 ($P<0.05$) difference was observed between both vaccinated groups at 28 doa (Fig. 4c).

299 **Protection**

300 After challenge, no clinical signs were observed in either vaccinated groups. In the
301 unvaccinated group, respiratory signs such as coughing, sneezing, head shaking, tracheal

302 rales and nasal discharge were observed until 5 dpc. The highest percentage of ciliary
303 protection (97%) was observed in group II, followed by group I (89.75%). The unvaccinated
304 challenged group (group III) showed little protection (12%) compared to the vaccinated
305 challenged groups.

306 Viral RNA loads, in all tracheal samples collected, were significantly higher ($P<0.05$)
307 (4.416 log REU RNA) in the unvaccinated challenged group (III) compared to the vaccinated
308 groups (I and II) as measured by real time RT-qPCR, at 5 dpc. The vaccinated groups, I and II
309 showed mean log REU of viral RNA of 1.016 and 0.555, respectively, with no significant
310 difference between these groups (Fig. 6). Overall viral RNA in the kidney samples of all the
311 groups were low compared to tracheal samples. The viral RNA load in kidneys in group III
312 was significantly higher ($P<0.05$) than in group II, whereas, group I showed no significant
313 difference ($P<0.05$) in log REU of viral RNA with either of group II and III.

314 Histopathological lesions in tracheas and kidneys were induced by challenge virus in
315 all the groups at 5 dpc. Marked histopathological changes occurred in group III (non-
316 vaccinated group) with mean scores of 10.2 and showed significant difference with group II
317 ($P<0.05$), but not with group I (Fig. 7). The mean lesion scores for kidneys in group III was
318 significantly higher ($P<0.05$) than group II, whereas, group I showed no significant
319 difference ($P<0.05$) in mean lesion scores with either group II and III. However, overall
320 mean lesion scores in kidneys were low compared to mean tracheal lesion scores.

321

322 **DISCUSSION**

323 The vaccination regime, chosen in this study for group I, is based on the research
324 demonstrating that improved protection was seen when two vaccines used were of different
325 serotypes (27, 32). They also emphasized that the vaccination programme used in their
326 experiments may not protect the respiratory tract against challenge with every new IBV

327 serotype to emerge. It is also evident that despite the use of Mass type vaccine at day 0,
328 followed by 793B type vaccine at 14 doa (same as in group I in this study), significant
329 number of new IBVs are still emerging under field conditions e.g. QX, IS/885/00, IS/1494/06
330 and most recently Q1. Therefore, in order to optimize the use of currently available vaccines,
331 to achieve better immunity and to assess protection against newly emerged Q1 strain, the
332 vaccination regime for group II was also included in this work.

333 At 1-day-old, chicks had high ELISA anti-IBV antibody titres in all groups, which
334 declined and dropped to below the cut-off point by 14 doa. In the groups that received the
335 vaccine at one day old, these low antibody levels could result from the partial neutralization
336 of the vaccine virus in the target tissues by the maternal antibodies present in the broilers at
337 that age, with a consequently low replication of the vaccine virus and poor stimulation of the
338 humoral response (16, 33, 34). Later, after the second vaccination at 14 doa, an increase in
339 the antibody titres was observed until 28 doa (day of challenge) in groups I and II with no
340 significant difference in antibody levels between these vaccinated groups ($P<0.05$). HI
341 antibody levels declined by 14 doa against the homologous and heterologous virus antigens,
342 showing similar patterns to declining ELISA titres. Interestingly, by 28 doa, there was no
343 significant difference between vaccinated groups I and II in terms of the level of antibodies to
344 M41, whereas, the HI titres to 793B and Q1 were significantly higher in group II than group I
345 ($P<0.05$). The role of antibody in the control of IBV infection remains controversial as
346 workers have shown that circulating antibody titres did not correlate with protection from
347 IBV infection (35-37). However, other studies demonstrated the importance of humoral
348 immunity in disease recovery and virus clearance (38, 39). In our study, as expected, the
349 higher HI titres were obtained using antigen homologous to vaccine strains. However, the
350 chicks also appeared to be protected against heterologous challenge. This could be due to the

351 presence of local immunity of the upper respiratory tract, induced by vaccination thus
352 reducing the replication of challenge virus.

353 The role of IgA antibodies is important for mucosal immunity to IBV and its presence
354 in tears following IBV antigen inoculation has been reported earlier (38, 40). In this study, a
355 gradual increase in IgA levels were observed in tears for both vaccinated groups during the
356 first two weeks after vaccination. These results are in agreement with previous research
357 reporting similar kinetics of lachrymal fluid IgA production to H120 vaccination (20, 41). In
358 addition, after the second vaccination, lachrymal IgA levels decreased in both vaccinated
359 groups, though, the levels in group II remained significantly higher ($P < 0.05$) than-group I.
360 This observation may indicate a decrease of lachrymal IgA levels after the second
361 vaccination is most likely due to partial neutralization of the anti-IBV IgA. A sharp decrease
362 of IgA-IBV in vaccinated chicks was also observed after challenge (42). In addition, no
363 significant rise in specific lachrymal IgA of vaccinated chickens was detected after
364 subsequent challenge with Ark-IBV isolate, explaining the probable role of neutralizing
365 antibodies in the lachrymal fluid at the time of challenge (43).

366 IBV-specific IgA can also be found in tracheal washes after an infection with strain of
367 IBV M41 (39, 40). In this study, the pattern of IgA in tracheal washes in both vaccinated
368 groups I and II was closely parallel, reaching peak at 7 doa and thereafter, declining till 28
369 doa, suggesting a short duration of the local humoral immunity in the trachea. Although
370 there have been conflicting reports on the relative concentrations of IgA in the avian
371 respiratory tract (44-46), our results are consistent with Hawkes *et al* (1983), which showed
372 IgA antibodies in tracheal washes only at day 7 after vaccination (47). Interestingly, in both
373 vaccinated groups, the second vaccination did not cause any rise in tracheal IgA level.
374 Similar findings have also been reported, revealing that the revaccination with homologous

375 IBV (M41 or H strains) (45), and secondary M41 IBV exposure (39), did not induce the
376 secondary secretory antibody response in tracheobronchial washings.

377 Consistent with the notion that CMI is protective against IBV (18, 48), we next sought
378 to study the level of systemic and local cellular immune responses. CD4⁺ cells may directly
379 produce antiviral cytokines, which increases B cell activity and promotes the proliferation,
380 maturation, and functional activity of CD8⁺ CTLs, which plays a critical role in controlling
381 IBV infection (49, 50). The ratio of CD4⁺:CD8⁺ has been widely shown to be indicative of
382 the general immune system status (51, 52). In this study, the CD4⁺:CD8⁺ ratio showed no
383 significant ($P<0.05$) variation among the groups till 14 doa. Nevertheless, the ratio at 28 doa
384 was found significantly higher ($P<0.05$) in group II than in groups I and III indicating that
385 second vaccination at 14 day-old in group II has probably enhanced the cellular immunity by
386 promoting the differentiation and proliferation of CD4⁺ cells in peripheral blood. There is no
387 specific data regarding the effects of different IBV vaccination on CD4⁺:CD8⁺ ratio in
388 peripheral blood so as to compare the present findings, however, Yohannes *et al.* (2012) have
389 reported significantly ($P<0.05$) higher CD4⁺:CD8⁺ ratio in IBV infected chicks than in the
390 controls (53). In addition, the high CD4⁺:CD8⁺ ratio has also been associated with increased
391 humoral incompetence in chickens, as a low CD4⁺:CD8⁺ ratio and a reduced response
392 against sheep red blood cells have been reported earlier (54). In this study, significantly
393 ($P<0.05$) higher HI titres at 28 doa to 793B and Q1 in group II than group I could be
394 attributed to the high CD4⁺:CD8⁺ ratio in that group at that time point. However, the
395 significance of this in relation to protection remains to be determined.

396 The results of IHC in tracheal tissue showed that the number of CD4⁺ lymphocytes
397 started increasing from 4 doa in both vaccinated groups, as compared to the control. At 28
398 doa, no significant difference was reported between the vaccinated groups. The CD8⁺ cells
399 subpopulation in both vaccinated groups started to increase significantly ($P<0.05$) at 4 doa,

400 reaching peak at 7 doa and then declining in number until 14 doa, suggesting that infiltration
401 and recruitment of these cells occurs in the first two week of initial IBV vaccination. Similar
402 to the findings of the present study, in the trachea, CD8+ cells recruitment in response to
403 infection were at a maximum by 7 days post infection (dpi) and CD4+ cells were not
404 recruited until 5 dpi. This was reported by Dhinakar *et al* (1996) (55). This work also showed
405 an overall higher infiltration of CD8+ cells in numbers compared to CD4+ cells in both
406 vaccinated groups. This observation is consistent with a previous study (55), where CD8+
407 cells were also found to predominate compared to CD4+ cells in trachea after IBV infection.
408 Moreover, the current study also documents significantly higher number of CD8+ cells in
409 vaccinated chicks of group II compared to group I on 21 and 28 doa, respectively. The IgA-
410 bearing B cells in vaccinated groups reached peak at 14 doa, however, the number of these
411 cells were significantly higher in group II in comparison to group I at 21 and 28 doa
412 ($P<0.05$). This pattern of recruitment of B cells later than either class of T cells is in
413 accordance with earlier studies (56, 57) who contended that local immunity against IBV is
414 mediated mainly by T-cells.

415 In this study, following the Q1 challenge, ciliary protection was higher in group II,
416 vaccinated with mixed H120 and CR88 vaccines at day-old, than in group I, vaccinated at
417 day old with H120 alone. Furthermore, the results of RT-qPCR showed that the viral RNA
418 load at 5 dpc in the trachea, was higher in group I than group II although the difference was
419 not statistically significant ($P<0.05$). In agreement with this, the scores of histopathology in
420 the trachea showed that the damage caused by the Q1 was higher in group I than II and
421 showed no significant difference in mean lesion scores with either of group II and III. On the
422 basis of these tracheal histopathological assessment, chickens in group II were better
423 protected compared with those in group I and this better protection might be attributed by
424 various factors including those discussed below.

425 Although the anti-IBV ELISA antibody titre results indicated that there was no
426 significant difference between the two vaccinated groups at the day of challenge, group II
427 showed higher ciliary protection than group I. This observation is consistent with previous
428 studies which have shown that circulating antibody levels were of minor importance in the
429 protection of the respiratory mucosa against IBV challenge (14, 44).

430 From our results, it appears that such overall higher protection could be due to
431 significantly higher levels of CD8+ cells in the tracheal tissues in group II than group I at day
432 of challenge. Previous study have shown that CD8+ cells are important contributors to viral
433 clearance in respiratory virus infections, utilizing contact-dependent effector functions, IFN- γ
434 and tumour necrosis factor- α (58). Therefore, we may speculate that the group II's higher
435 CD8+ cell reaction than the group I could have contributed to the faster viral clearance after
436 challenge with Q1, explaining the differences between the vaccinated groups in their tracheal
437 protection. This possible explanation agrees with other studies that emphasized the
438 involvement of local CD8+ cells in the infection of chickens with respiratory pathogens, such
439 as Newcastle disease virus (59) and *Mycoplasma gallisepticum* (60). Additionally, group II's
440 higher levels of IgA in lachrymal fluid, compared to group I, could reduce the tracheal
441 histopathological damage which also corroborates the hypothesis that the traditional role of
442 IgA is to prevent pathogen entry at mucosal surfaces and neutralize virus in infected
443 epithelial cells (61). IBV-specific IgA antibodies in lachrymal fluid were correlated with
444 resistance to IBV reinfection (38, 40, 42). Our results are in agreement with a recent study by
445 Okino *et al* (2013), in which the authors concluded that IBV IgA antibodies in lachrymal
446 secretions and the expression of granzyme-A and CD8 genes in tracheal tissues after H120
447 vaccination, provides a reliable approach to monitor immune protection status in the trachea,
448 as shown by examination for cilliositosis, histopathology and viral replication (20). For our
449 study, we aimed to stain for a variety of cell-surface markers and thereby identify the T cell

450 populations infiltrating the trachea. This provides further information about the role of cell-
451 mediated immunity in protection given by different live IBV vaccination regimes against a
452 novel IBV Q1 challenge.

453 The results of RT-qPCR and scores of histopathology in the kidneys showed that the
454 damage caused by the Q1 was higher in group I than II, and showed no significant difference
455 in the mean lesion scores with either of group II and III. Specific cytotoxic T lymphocytes
456 have been shown to be important for the systemic clearance of nephropathogenic IBV and
457 reduction of kidney lesions (15). A plausible explanation is that a higher CD8+ cells response
458 in the tracheal tissues (the portal of entry of challenge virus) in group II compared to group I
459 could have prevented the challenge virus becoming viraemic thus failing to reach the kidneys.
460 This provided an efficient prevention of kidney infection, as measured by viral RNA load and
461 histopathological lesion scores in renal tissue.

462

463 **CONCLUSIONS**

464 Chicks vaccinated with H120 and CR88 at day-old, followed by CR88 at 14 doa, showed
465 significantly higher CD8+ responses in the trachea and higher lachrymal IgA levels compared
466 to those vaccinated with H120 alone. In terms of ciliary protection against Q1, though both
467 vaccinated groups were protected, the combined vaccination of H120 and CR88 of day-old
468 chicks, followed by CR88 at 14 doa, showed higher ciliary protection and less RNA load in
469 trachea and kidneys, wherein histopathological lesions are reduced. This study highlighted
470 the potential modulation of chick immune response with the use of currently available live
471 vaccines so that better protection against variant IBVs can be afforded.

472

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475

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- 645

646 **Fig. 1.** Anti-infectious bronchitis virus (IBV) antibody titres of the different groups
647 vaccinated with a live H120 alone (group I) or in combination with CR88 (group II) at day-
648 one. Both groups were again vaccinated with CR88 at 14 days of age. One group (group III)
649 was kept as control. Where values were significantly ($P<0.05$) different these are shown with
650 different letters and all other values were not significantly ($P<0.05$) different between the
651 groups on those sampling points.

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654 **Fig. 2.** Detection of IgA production using ELISA in (a) tears (b) tracheal wash of chickens
655 (n=5 per group) vaccinated with a live H120 alone (group I) or in combination with CR88
656 (group II) at day-one. Both groups were again vaccinated with CR88 at 14 days of age. One
657 group (group III) was kept as control. The IgA antibody levels in tears and tracheal wash
658 from control chickens (group III) remained below the detectable level. Asterisks indicate
659 values between the two vaccine groups were significantly different ($P<0.05$) on those time
660 point. Error bars indicate standard error of the mean.

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663 **Fig. 3.** The ratio of CD4⁺:CD8⁺ analyzed by flow cytometry in peripheral blood of chickens
664 vaccinated with a live H120 alone (group I) or in combination with CR88 (group II) at day-
665 one. Both groups were again vaccinated with CR88 at 14 days of age. One group (group III)
666 was kept as control. Depicted are the mean values (n=5 per group) and one standard error.
667 Where values were significantly ($P<0.05$) different these are shown with different letters and
668 all other values were not significantly ($P<0.05$) different between the groups on those time
669 point.

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672 **Fig. 4.** Immunohistochemical detection of CD4⁺ cells in group II at 28 days of age (B),
673 CD8⁺ cells in group II at 28 days of age (C), IgA-bearing B-cells group II at 28 days of age
674 (D), in tracheas of chickens vaccinated with live H120 alone (group I) or in combination with
675 CR88 (group II) at day-one. Both groups were again vaccinated with CR88 at 14 days of
676 age. One group (group III) was kept as control (A). Magnification (400x). Arrows indicate
677 positive cells.

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680 **Fig. 5.** Summary of CD4⁺ cells (a), CD8⁺ cells (b), and IgA-secreting cells (c) determined by
681 immunohistochemical staining in the trachea of chickens vaccinated with a live H120 alone
682 (group I) or in combination with CR88 (group II) at day-one. Both groups were again
683 vaccinated with CR88 at 14 days of age. One group (group III) was kept as control. Depicted
684 are the mean values (n=5 per group) and one standard error. Where values were significantly
685 ($P<0.05$) different these are shown with different letters and all other values were not
686 significantly ($P<0.05$) different between the groups at those time points.

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695 **Fig. 6.** Quantification of infectious bronchitis virus (IBV) expressed as log REU of RNA, in
 696 trachea and kidney measured by real time RT-PCR after 5 dpc from chickens experimentally
 697 challenged at 28 days of age with Q1 strain of IBV (n=10 per group). The chickens were
 698 previously vaccinated with a live H120 alone (group I) or in combination with CR88 (group
 699 II) at day-one. Both groups were again vaccinated with CR88 at 14 days of age. One group
 700 (group III) kept as control received sterile water. Significant differences between the groups
 701 were detected by Kruskal-Wallis test followed by Dunn's mean test indicated with different
 702 letters ($P<0.05$).

703

704 **Fig. 7.** Means of histopathological scores of lesions in trachea and kidney samples after 5 dpc
 705 from chickens experimentally challenged at 28 days of age with Q1 strain of infectious
 706 bronchitis virus (n=10 per group). The chickens were previously vaccinated with a live H120
 707 alone (group I) or in combination with CR88 (group II) at day-one. Both groups were again
 708 vaccinated with CR88 at 14 days of age. One group (group III) kept as control was inoculated
 709 with sterile water. Significant differences between the groups were detected by Kruskal-
 710 Wallis test followed by Dunn's mean test indicated with different letters ($P<0.05$)

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715 **Table 1:** Study design showing groups, vaccine and vaccination regimes. At 28 day of age,
 716 10 chicks from each group were challenged with a virulent IBV Q1.

IBV vaccine (dosage/chick in 100 μ l)	Group/days of age					
	I		II		III	
	0	14	0	14	0	14
H120 (3.5 log ₁₀ EID ₅₀)	√		√	√		
CR88 (4.25 log ₁₀ EID ₅₀)		√		√		
Sterile water (SW)					√	√

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719 **Table 2.** Geometric mean anti-IBV HI antibody titre (\log_2) in serum of chickens vaccinated with live H120 alone (group I) or in combination
 720 with CR88 (group II) at day-one. Both groups were again vaccinated with CR88 at 14 days of age. One group (group III) was kept as control.
 721 Significant differences between the groups (n=5 per group) for each homologous as well as heterologous antigen for each interval are
 722 represented by different letters ($P<0.05$)

723

HI antigen	Groups	Days of age						724
		0	4	7	14	21	28	
M41	Group I	9.2±0.374 ^A	8.2±0.970 ^A	7.4±0.400 ^A	5±0.000 ^B	5±0.548 ^A	4±0.447 ^A	725
	Group II	9.2±0.374 ^A	8.8±0.374 ^A	7.4±0.400 ^A	6.4±0.510 ^A	5.4±0.400 ^A	4.4±0.400 ^A	726
	Group III	9.2±0.374 ^A	7.2±0.374 ^A	6.4±0.748 ^A	4.8±0.200 ^B	4±0.632 ^A	2±0.632 ^B	727
CR88	Group I	8.4±0.400 ^A	7.8±0.374 ^A	6.8±0.490 ^A	5±0.316 ^A	6.8±0.583 ^A	4.4±0.678 ^B	728
	Group II	8.4±0.400 ^A	8.4±0.245 ^A	6.8±0.374 ^A	5.8±0.200 ^A	7±0.548 ^A	8.2±0.583 ^A	729
	Group III	8.4±0.400 ^A	7.6±0.400 ^A	7.4±0.245 ^A	4.6±0.600 ^A	3.4±0.245 ^B	1.8±0.490 ^C	730
Q1	Group I	7±0.316 ^A	3±0.316 ^A	2.4±0.245 ^A	2.2±0.200 ^B	4.4±0.510 ^A	4.4±0.510 ^B	
	Group II	7±0.316 ^A	3±0.678 ^A	3.4±0.400 ^A	3.8±0.374 ^A	5±0.548 ^A	5.6±0.245 ^B	
	Group III	7±0.316 ^A	3.4±0.316 ^A	2.2±0.200 ^A	2±0.000 ^B	2±0.000 ^B	2±0.000 ^C	

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