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1 **Immunization of broiler chickens against *Clostridium perfringens*-induced necrotic**
2 **enteritis**
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Abstract

Necrotic enteritis (NE) in broiler chickens is caused by *Clostridium perfringens*. Currently, no vaccine against NE is available and immunity to NE is not well characterized. Our previous studies showed that immunity to NE followed oral infection by virulent rather than avirulent *C. perfringens*, and identified immunogenic secreted proteins apparently uniquely produced by virulent *C. perfringens*. These proteins were alpha-toxin, glyceraldehyde 3-phosphate dehydrogenase, pyruvate: ferredoxin oxidoreductase (PFOR), fructose 1,6-biphosphate aldolase, and a Hypothetical Protein (HP). The current study investigated the role of each of these proteins in conferring protection to broiler chickens against different severities of oral infection challenge with virulent *C. perfringens*. Genes encoding these proteins were cloned and purified as histidine-tagged recombinant proteins from *E. coli* and used to immunize broiler chickens intramuscularly. Serum and intestinal antibody responses were assessed by ELISA. All proteins significantly protected broiler chickens against a relatively mild challenge. In addition, immunization with alpha-toxin, HP and PFOR also showed significant protection against a more severe challenge. When primed with alpha-toxoid and boosted with active toxin, birds immunized with alpha-toxin showed the greatest protection against a severe challenge. The serum and intestinal washings from protected birds had high antigen-specific antibody titers. Thus, we conclude that there are certain secreted proteins, in addition to alpha-toxin, that are involved in immunity to NE in broiler chickens.

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44 Necrotic enteritis (NE) is an economically important enteric disease of chickens caused
45 by *Clostridium perfringens*. The disease is usually controlled by antimicrobial drugs
46 administered at prophylactic doses either in water or feed. However, there is concern
47 about the routine prophylactic use of antimicrobial drugs in food animal production
48 because of their contribution to antimicrobial resistance problems. If antimicrobial drugs
49 were banned for such purposes in North America, there might be an increase in NE in
50 broiler flocks, as has happened in Scandinavia (12).

51 Although vaccination offers an alternative approach to antimicrobial drugs in control of
52 the disease, very little is known about immunity to NE. However, there has been
53 considerable work on immunity to *C. perfringens* in other circumstances, since it is a
54 cause of gas gangrene in people. This has identified the alpha-toxin, a phospholipase C
55 exoenzyme, both as a major virulence factor and as an important protective immunogen
56 (5, 30, 34). In addition, based on naturally occurring antibodies or maternal vaccination,
57 some studies suggest that antibodies to alpha-toxin are important in immunity to NE in
58 chickens (10, 19). However, the role of alpha-toxin or any other protein in immunity to
59 NE in chickens remains to be demonstrated, and one study has shown the immunizing
60 effects of alpha-toxin minus mutants (32). A recent study also demonstrated that an
61 alpha-toxin minus mutant produced NE experimentally in chickens, demonstrating that
62 factors other than alpha-toxin are important in the pathogenesis of NE (14).

63 Recent studies from this laboratory showed that the immunizing ability to protect
64 against NE was associated with infection by virulent rather than with avirulent *C.*
65 *perfringens* (32). Several proteins apparently uniquely expressed by virulent, protective
66 *C. perfringens* that reacted to serum and intestinal antibodies from infection-immunized

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67 birds were identified by mass spectrometry (15). These secreted proteins were alpha-
68 toxin, glyceraldehyde 3-phosphate dehydrogenase (GPD), pyruvate: ferredoxin
69 oxidoreductase (PFOR), fructose 1,6-biphosphate aldolase (FBA) and a Hypothetical
70 Protein (HP). Based on these findings, the objective of the current study was to
71 investigate the role of each of these proteins in immunizing broiler chickens against
72 experimental challenge of varying severity with virulent *C. perfringens*.
73

74 Materials and Methods

75 **Cloning, overexpression and purification.** *Escherichia coli* strains were used to clone
76 and express the genes of interest; *E. coli* DH5α (*rec*A *lac*ZΔM15) (Stratagene, La Jolla,
77 CA) was used as the host for plasmid construction, and *E. coli* BL21-Star DE3 (F^- *omp*T
78 *hsd*S_B(r_B-m_B-) *gal dcm rne*131) (Invitrogen, Carlsbad, CA.) was used for over-expression
79 of Histidine-tagged fusion proteins. These strains were grown in Luria-Bertani (LB)
80 medium at 37ºC and when required, kanamycin was added to the medium at a
81 concentration of 50 µg/ml. The chromosomal DNA of the virulent, protective, *C.*
82 *perfringens* strain CP4 was used as the source of DNA for expression of secreted
83 antigens. PCR amplifications were performed using Platinum PCR SuperMix High
84 Fidelity (Invitrogen, Burlington, ON) and specific primers described in Table 1. After
85 purification (PCR Purification Kit, Qiagen, Mississauga, ON), the PCR products (alpha-
86 toxin, HP, GPD, FBA and tPFOR) were cloned into vector pET28a (N' and/or C'
87 terminal His tag vector, Km^R from Novagen Inc., Madison, WI) to generate proteins
88 fused with histidine residues (6-His). The resulting plasmids were introduced into *E coli*
89 BL21 Star (DE3), following the manufacturer's instructions. The nucleotide sequences of

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90 the cloned PCR products were verified by sequencing both strands. Expression of
91 recombinant proteins by *E. coli* was induced by Isopropyl-ß-D-thiogalactopyranoside
92 (IPTG) at a final concentration of 1 mM**.** Histidine tagged proteins were purified under
93 native conditions using affinity chromatography on nickel-nitrilotriacetic acid (Ni-NTA)
94 agarose following the manufacturer's instructions (Qiagen). Briefly, when the proteins
95 were expressed as soluble proteins, bacterial pellets were resuspended in a lysis/ binding
96 buffer (50mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole) containing lyzozyme (1
97 mg/ml) and incubated for 60 min on ice. The bacterial cells were lysed using a French
98 pressure cell (3-4 cycles of 1000 psi). The supernatant was collected by centrifugation
99 and added to Ni-NTA agarose. The washing and elution steps were performed using
100 buffers containing increasing concentration of imidazole (20 mM to 250 mM). Finally
101 imidazole was removed from the eluted material by dialysis against phosphate buffered
102 saline, pH 7.2, (PBS) and the recombinant proteins were concentrated using Amicon
103 filter -10kD (Millipore, Billerica, MA) and the protein concentration was determined
104 using PlusOne™ 2-D Quant kit (Amersham Biosciences, San Francisco, CA).
105 **SDS-PAGE and Western Immunoblot.** Purified recombinant proteins were separated
106 by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-
107 PAGE) in a 12.5% acrylamide gel under denaturing conditions as described by Laemmli
108 (16). Proteins were transferred to nitrocellulose membrane of 0.45 µm pore size using a
109 mini-gel transfer assembly (Bio-Rad Laboratories, Hercules, CA). After completion of
110 transfer, non-specific binding sites on the membranes were blocked for 1 h with blocking
111 buffer containing 1% casein (Bio-Rad Laboratories) and incubated with immune sera
112 collected from infection-immunized birds in a previous study (32) for 1 h at 1:1000

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113 dilution. Goat anti-chicken IgY (H+L) (Cedarlane Laboratories, Hornby, ON) was used
114 as secondary antibody at 1:2000 dilution. The blots were developed and specific
115 immunoreactive protein bands were visualized using an alkaline phosphatase-conjugated
116 substrate kit (Bio-Rad Laboratories).

117 **Immunization and challenge.** Experiments with chickens and conditions for their use
118 were approved by the University of Guelph Animal Care Committee in accordance to the
119 Canadian Council on Animal Care's Guidelines. Commercial 1-day-old male White
120 Plymouth Rock broiler chickens (Bonnie's Chick Hatchery, Elmira, ON) were fed an
121 antibiotic-free chicken starter containing 20% protein for 13 days followed by a
122 formulated wheat-based grower feed, containing 28% protein (Arkell Research Station,
123 University of Guelph). Birds were immunized with purified recombinant proteins at
124 different dose levels intramuscularly in the pectoral muscle in a volume of 0.2 ml per bird
125 2-3 times with an interval of one week and challenged a week after the last immunization
126 when birds were 4 weeks old. For the experimental infection (challenge) of birds, virulent
127 *C. perfringens* (CP4) was grown in cooked meat medium (Difco) (CMM) for 24 h at
128 37°C. Fluid thioglycolate medium (Difco) was then inoculated with a 3% (v/v) inoculum
129 from the *C. perfringens* infected CMM and incubated at 37°C for 24 h. The growth at
130 24 h was $\log_{10}$ 8.24 ± 0.09 *C. perfringens* CFU/ml. The inoculated FTG was then mixed
131 with feed at a ratio of 2:1 (v/w). Inoculated feed was prepared freshly twice per day and
132 fed to chickens that were fasted for 20 h prior to challenge.

133 The general experimental design is summarized in Table 2. Quil-A (Superfos Biosector,
134 Vedbaek, Denmark) was used as an adjuvant to immunize chickens (50 µg/ bird/
135 injection) and the unimmunized controls in each experiment received only Quil-A

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136 followed by a challenge similar to immunized groups. All the proteins were tested for
137 their protective ability against a gradient of severity of challenge (mild-moderate-severe).
138 A "mild" challenge (Experiment 1) was produced by a duration of challenge of 3 days;
139 the mildness of challenge was confirmed by the lesions produced in non-immunized birds
140 were. A "moderate" challenge (Experiment 2) was produced by duration of challenge of
141 5 days, and when birds were fed a fixed amount of feed that sometimes ran out before the
142 next 12-hourly feeding; the moderateness of challenge was confirmed by lesion scores of
143 the non-immunized birds. A "severe challenge" (Experiment 3) was produced by
144 challenge for 5 days and by ensuring that birds had infected feed constantly available; the
145 severity of challenge was confirmed by the lesion scores of non-immunized birds. In
146 Experiment-4A, the challenge was considered 'mild-moderate' since birds in these
147 groups that received virulent *C. perfringens* for 3 days but were necropsied on the day 6
148 and were found to have higher lesion scores compared to those that were challenged for 3
149 days and necropsied on day 4. Alpha-toxin was used in both an active and a toxoid form
150 to immunize birds and toxoiding was done following a previously described protocol
151 (11). Briefly, the purified toxin was incubated with 0.2% formalin and 0.1 M L-lysine at
152 30°C for a period until its activity was completely lost as confirmed on 5% egg-yolk agar
153 plate assay. In all experiments, the number of birds in each group was between 10 and 20
154 and all birds were identified individually. Blood was collected from the wing vein from
155 all the groups at three times: pre-immunization (day- 0), mid-experiment (day- 10) and
156 pre-challenge (day- 20). Intestinal washings were collected using PBS at necropsy.

157 **Necropsy.** Chickens were euthanized with carbon dioxide gas and their small intestines
158 (duodenum to ileum) examined for grossly visible lesions. Any chickens that had

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159 reached a pre-determined severity of clinical illness prior to necropsy were euthanized
160 and later necropsied. Intestinal lesions in the small intestine (duodenum to ileum) were
161 scored as follows: 0 = no gross lesions; 1 = thin or friable wall or very mild and
162 superficial generalized inflammation; 2 = focal necrosis or ulceration; 3 = large patches
163 of necrosis; 4 = severe extensive necrosis; 5 = chickens that died during experiment,
164 having 4+ lesions (25). Blind scoring was employed to avoid the scorer bias.

165 **Measurement of the antibody titers in chicken sera and intestinal washings.** The
166 specific antibody titers were determined by the end point dilution method using an
167 enzyme-linked immunosorbent assay (ELISA). Microtiter plates (Immulon-2, Chantilly,
168 VA) were coated with recombinant proteins (5µg/ml in 0.1 M carbonate buffer pH 9.6)
169 for 60 min at 37°C followed by an overnight incubation at 4°C. After blocking coated
170 plates for 60 min at 37°C with PBS containing 3% of bovine serum albumin (BSA
171 Sigma), sera from immunized birds, along with their concurrent controls were serially
172 diluted in PBS containing BSA 1% and incubated for 2 h with recombinant protein-
173 coated plates at room temperature. After washing with PBS containing Tween 20 0.1%
174 (PBST), alkaline phosphatase (AP)-coupled goat anti-chicken IgY (H+L) (1:5,000 in
175 PBS-Tween 20 0.1%-BSA 1%) was added to the microplates and incubated for 60 min
176 at room temperature. After extensive washing with PBST, color reaction was developed
177 using alkaline phosphate substrate kit (Bio-Rad Laboratories) following the
178 manufacturer's instructions. The reaction was stopped by adding 0.4 M NaOH. The
179 absorbance was measured at 405 nm in an ELISA spectrophotometer. The specific
180 antibody titer of immune serum was expressed as the reciprocal of the serum dilution

(log_2 OD) that gave an A_{405} value above the cut-off, defined as twice the absorbance value of the un-immunized and mock control wells run in duplicates.

Intestinal antibody response was also measured by ELISA following the procedure described above for serum. Intestinal washings from at least 10 chickens per group were pooled and the total protein content was measured using PlusOne™ 2-D Quant kit (Amersham Biosciences) and used as source of primary antibody after keeping the protein content of the initial dilution (1:10) constant across all the groups. Alkaline phosphatase conjugated goat anti-chicken IgY and IgA were used as secondary antibodies at dilutions of 1:4000 and 1:2000 respectively. The end-point titers were determined as described above for serum ELISA.

Statistical analysis. Statistical analysis was performed to determine whether there was a significant difference between the number of birds with lesions from immunized groups and birds from the unimmunized, vehicle-only controls. A two-tailed Fisher's exact test determined whether the two groups differed in the proportion with which they fell into the two classifications of either lesions or no lesions, under the null hypothesis that the proportions were the same. Data was analyzed in a 2×2 contingency table with the unimmunized control group in one column and immunized groups in the other column. Lesion scores were ranked from 0 to 5+, however, a “protective” response was given to birds with ≤1+ lesions. The null hypothesis was rejected at $\alpha = 0.05$. For serum ELISA, a one-way ANOVA was used to determine significant ($p \leq 0.01$) differences in the antibody titers between pre-immunized and immunized birds across all the groups. Statistical analysis could not be made on intestinal ELISA, since the washings collected were pooled per group.

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204 ## Results

205 **Cloning, expression and purification.** All five genes selected for the immunization
206 study were successfully cloned, expressed and purified to homogeneity. However, alpha-
207 toxin appeared to be toxic to the host *E. coli* cells such that it could not be obtained in
208 sufficient quantity for immunization studies. Hence, commercially available purified
209 alpha-toxin (Sigma Laboratories) (and a toxoided version) was used to immunize birds.
210 Hypothetical Protein (190 kDa) was found to be cleaved upon expression into two bands
211 of 90-100 kDa. Attempts to express the entire protein by using different *E. coli*
212 expression hosts were unsuccessful. Since both bands reacted strongly to anti-histidine
213 antibodies as well as to immune sera collected from infection-immunized birds (Figure 1)
214 from a previous study (32), both bands were further purified in large quantities and used
215 in immunization.

216 Cloning of the *pfor* gene was not successful despite several attempts. However, a
217 portion of the gene that encoded a truncated protein (tPFOR) of 67 kDa size that
218 contained the Iron- Sulphur (Fe-S) active sites of this enzyme was successfully cloned
219 and purified in large quantities.

220 All the recombinant proteins purified from *E. coli* were visualized by Coomassie
221 staining and their reactivity to anti-histidine antibodies as well as immune serum was
222 confirmed by Western blot in at least three separate experiments (Figure 1).

223 **Immunization experiments.** In Experiment-1, HP, GPD, tPFOR and FBA showed
224 significant protection against a mild challenge (Table 3), of which HP showed the

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225 greatest protection. Immunization with crude culture supernatant that contained all
226 secreted proteins including those purified also showed significant protection. Alpha-
227 toxoid did not protect birds against challenge.

228 In Experiment-2, HP alone showed significant protection against a moderate challenge,
229 whereas GPD, tPFOR and FBA did not (Table 4). Immunization (mock) with an
230 unrelated purified recombinant fusion protein showed a mean lesion score similar to that
231 of unimmunized controls. The increased mean lesion score of controls compared to
232 Experiment-1 appeared to reflect increased duration of challenge.

233 In Experiment-3, birds that received two initial injections of alpha-toxoid but a third
234 injection with active alpha-toxin showed the greatest protection against a heavy challenge
235 (Table 5). Birds immunized with either HP or tPFOR also showed significant protection
236 against severe challenge. Although birds immunized with GPD, FBA and the
237 combination of GPD and HP had mean lesion scores lower than non-immunized controls,
238 no statistically significant protection was observed.

239 In Experiment- 4A, FBA immunized birds showed significant protection against a mild-
240 moderate challenge compared to unimmunized controls. Mean lesion scores of
241 unimmunized controls of Experiment- 4B were comparable to the scores of unimmunized
242 controls in Experiment-3 that also received a severe challenge.

243 A visual summary of mean lesion scores of birds from all immunized groups that
244 received different doses of antigens and challenge across different experiments, together
245 with the concurrent unimmunized controls, is shown in Figure 2.

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246 **Antibody titers in chicken sera and intestinal washings.** All the proteins used to
247 immunize birds in the immunization experiments described produced significant antigen-
248 specific serum antibody titers (Figure 3) compared to pre-immunization titers. Birds
249 immunized twice with a higher antigen quantity had lower titers than birds immunized
250 three times with a lower amount. Protection in Experiment 3 was not as marked as in
251 Experiment 1, although antibody titers were generally similar. This difference was
252 attributed to a difference in severity of challenge. There was a discrepancy between
253 antibody titers to alpha-toxin and protection, since either alpha-toxoid or toxin
254 immunized but non-protected birds in Experiments-1 and 4 respectively, had higher titers
255 than toxoid/ active toxin immunized birds that were significantly protected in
256 Experiment-3.

257 Intestinal antibody responses to all the proteins used for immunization showed higher
258 IgY than IgA titers (Figure 4), but markedly lower titers of both isotypes than observed in
259 serum of birds. However, the IgY and IgA titers were generally similar in birds
260 immunized with alpha-toxoid or toxin (Experiments- 1, 3 and 4).

261 ## Discussion

262 This study has shown, for the first time, that a degree of immunity to NE in broiler
263 chickens can be produced by immunization with several different secreted *C. perfringens*
264 proteins, and that the degree of protection is a function of the severity of the challenge.
265 All the proteins used in immunization, including alpha-toxin, showed significant
266 protection depending on the severity of challenge. It seems that protection against NE lies
267 in the secreted component of *C. perfringens* since immunization with crude culture

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268 supernatant that included all proteins tested in the current study largely protected birds
269 against challenge. Of the five secreted proteins used for immunization, three proteins,
270 namely alpha-toxin, HP and tPFOR, significantly protected chickens against a heavy
271 challenge, whereas the other two proteins, GPD and FBA, significantly protected only
272 against mild challenge. Nevertheless a degree of protection was apparent with these latter
273 proteins even against severe challenge.

274 The role of alpha-toxin in immunity to NE has been suspected but not previously clearly
275 demonstrated. Priming with alpha-toxoid and boosting with active toxin showed the best
276 protection, whereas immunization with three injections of either alpha-toxoid or of active
277 toxin did not protect (Tables 3, 6). The failure of active toxin to protect birds against a
278 heavy challenge may have resulted from the toxin's activity on immune system cells. The
279 failure of alpha-toxoid to protect in the Experiment-1 may be the result of a degradation
280 effect of toxoiding on the protein that was observed on SDS-PAGE gel (data not shown).
281 However, it is clear from Figure 3 that toxoiding was adequate to induce antibodies
282 sufficient for the birds to tolerate the active toxin given as a booster in the Experiment-3.
283 The findings from the present and an earlier study (15) suggest that antibodies to
284 conformational (rather than linear) epitopes of alpha-toxin are critical in protection
285 against NE. Achieving conformational but non-toxic epitopes in a vaccine may prove
286 challenging, (1, 7, 33). , Although many studies have emphasized the importance of non-
287 toxic C-terminal domain in protection against experimental gas gangrene (5, 30, 34),
288 some have shown the neutralizing epitopes to be on the N-terminus (18). It seems likely
289 that the positioning of protective, neutralizing, conformational epitopes of alpha-toxin is

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290 subtle. For this reason, other immunogens such as those identified here may be more
291 feasible candidates for immunization.

292 Perfringolysin O is a potent hemolytic cytolysin that mediates necrosis in the
293 pathogenesis clostridial gas gangrene (29) and an important protective immunogen in
294 mouse and guinea pig gas gangrene models (8). Our previous study suggested its possible
295 role in NE immunity in broiler chickens (15). In the current study, the purified alpha-
296 toxin (Sigma Laboratories) used to immunize birds had traces of perfringolysin O,
297 identified by us using mass spectrometry (data not shown). However, the relative
298 amounts in the otherwise apparently pure toxin preparation (assessed by SDS-PAGE)
299 were not quantified. It is possible that the protection observed in alpha-toxoid/toxin
300 immunized birds (Table 5), can be partly attributed to perfringolysin O or even to traces
301 of other but undetected immunogenic proteins and that a synergistic effect on the
302 induction of neutralizing antibodies against both the toxins may have contributed to better
303 protection (2).

304 The observation that immunization with secreted proteins, other than alpha-toxin,
305 provides some immunity to birds against NE highlights the likely involvement of several
306 proteins in the pathogenesis of this infection. Both alpha-toxin and perfringolysin O are
307 regulated in *C. perfringens* by the VirR-VirS two-component regulon. (4, 26) a regulon
308 that also controls genes involved in energy metabolism such as FBA, as well as others
309 that may be indirectly involved in bacterial virulence (3, 13, 28). There is growing
310 evidence that certain enzymes such as GPD and FBA, that are conventionally regarded as
311 metabolic or "house-keeping" enzymes, may have a 'dual role' in both the pathogenesis
312 of, and immunity to, other infections (6, 9, 17, 20, 22-24, 35). Interestingly, a recent

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313 study showed that antibodies to FBA and GPD of *Streptococcus pneumoniae* showed an
314 age-dependent increased serum titers in children of different ages. Immunization of mice
315 with recombinant GPD and FBA showed significant protection against respiratory
316 challenge with virulent *S. pneumoniae* (17). A role for FBA in immunity to *Onchocerca*
317 *volvulus* has also been suggested (21). Similarly, PFOR, an enzyme crucial for anaerobic
318 energy metabolism, has been suggested to have a role in immunity to invasive amoebiasis
319 (31). Hypothetical Protein is a novel protein of *C. perfringens* of unknown function
320 identified in its genome (27) that may have protease activity (zinc- metallopeptidase)
321 based on the analysis of its protein structure (15). It will be of interest to determine
322 whether HP is a virulence determinant. It is apparent from the present immunization
323 study that, besides alpha-toxin, other proteins (HP, GPD, tPFOR and FBA) are important
324 in some aspects of host- pathogen interaction during the disease process. The recent
325 demonstration that alpha-toxin is apparently not essential in the NE pathogenesis (15),
326 supports the suggestion that other proteins are involved.

327 Alpha-toxoid/ toxin immunized- protected birds had lower antibody titers than toxoid-
328 immunized birds that were not protected, suggesting the importance of conformational
329 epitope- specific neutralizing antibodies in mounting a protective immune response. This
330 implies the importance of quality of response in protection. The intestinal antibody
331 response, as expected, was mainly IgY dominated since systemic immunization results in
332 more antigen- specific IgY than IgA (Figure 4) that reaches mucosal surfaces under
333 inflammatory or necrotic conditions of the gut allowing seepage of serum IgY at the site
334 of infection. It is also possible that a mucosal IgY response is more important in
335 immunity to *C. perfringens*-induced NE since a previous study showed weak reactivity of

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336 *C. perfringens* proteins to mucosal IgA in the intestinal washings collected from orally
337 infection-immunized birds (15). Immunization with HP, that significantly protected birds
338 against all severities of challenge doses, produced higher IgA titers in all three
339 experiments compared to other immunized groups. However, this association of IgA
340 titers to protection was not evident in either alpha-toxoid/ toxin or tPFOR immunized
341 groups that were also significantly protected birds against a heavy challenge in
342 Experiment- 3.

343 In conclusion, this is the first report that has demonstrated the immunizing ability of *C.*
344 *perfringens* secreted proteins including alpha-toxin in protecting against NE in broiler
345 chickens. It seems likely that some of the secreted proteins that appear important in NE
346 immunity also play a previously unsuspected role in the pathogenesis of the disease.
347 This study also suggests that conformational epitopes of alpha-toxin are important in
348 immunity and that antibody to alpha-toxin provides birds with better protection.
349 Nevertheless, there are other proteins that might be suitable vaccine candidates in
350 preventing this important disease.

351

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462 against the alpha-toxin of *Clostridium perfringens* protects mice against experimental gas
463 gangrene. Vaccine **11:**1253-1258.

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466 Table 1. List of primers used to amplify genes encoding proteins used in immunization

467 experiments

Gene	Sequences 5'-3'	Amplicon size (bp)
Alpha-toxin	Forward-ccgctcgagttgggatggaaaaattgat	1100
	Reverse-ccggaattctttatattataagttgaattt	
Hypothetical protein	Forward- ccgctcgaggaataagagaaaaatagcag	5400
	Reverse- ccgggtaccacgttaaataaatagaacat	
Glyceraldehyde 3-phosphate dehydrogenase	Forward- ccgctcgagggtaaaagtagctattaacgg	1000
	Reverse- ccgggtaccttagaaactaagcattttaaa	
Fructose 1,6-biphosphate aldolase	Forward- ccgcggatccatggcattagttaacgcaaa	900
	Reverse- ccgcctcgagagctctgtttactgaaccga	
Truncated pyruvate: ferredoxin oxidoreductase	Forward- ccgcctcgagcacttcattagaaccagttg	1600
	Reverse- ccgcggatcctagctaagtagtcttggtct	

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470 Table 2. Summary of experimental design

471

Experiment	Immunization Groups	Dosage of vaccine/ Bird	Frequency of administration	Oral challenge
1	VC, Sup[a], Alpha-toxoid, GPD, HP, FBA and tPFOR	20 µg	Three times; day 7, 14 and 21	3 days (mild)
2	VC, MC[b], GPD, HP, FBA and tPFOR	40 µg	Two times; day 7 and 14	5 days (moderate)
3	VC, Alpha-toxoid/ toxin[c], GPD, HP, tPFOR, combination of GPD and HP	20 µg	Three times; day 7, 14 and 21	5 days (severe)
4A	VC and FBA	20 µg	Three times; day 7, 14 and 21	3 days (mild-moderate)
4B	VC, Alpha-toxin[d] and FBA	20 µg	Three times; day 7, 14 and 21	5 days (severe)

472

473 VC- Vehicle-only controls, GPD- glyceraldehyde 3-phosphate dehydrogenase, tPFOR-

474 truncated pyruvate: ferredoxin oxidoreductase, FBA- fructose 1,6-biphosphate aldolase,

475 HP- Hypothetical protein, Sup- crude culture supernatent of virulent C. perfringens and

476 MC- mock-immunized controls

477 [a] Birds received 60 µg/inj of culture supernatant that was processed and concentrated

478 following a protocol described earlier (15).

479 [b] Birds were mock immunized with an unrelated protein that was cloned, expressed and

480 purified from *E. coli* in the same manner as *C. perfringens* related proteins.

481 [c] Birds received alpha-toxoid in the first two injections followed by active alpha-toxin in

482 the third.

ACCEPTED

483 [d] Birds in this group received three injections of alpha-toxin where in the first and the

484 third injections were with 20 μg and the second was reduced to 10 μg.

ACCEPTED

485 Table 3. Intestinal lesion scores of birds immunized with three injections intramuscularly,

486 then infected with a mild challenge by C. *perfringens*

Protein	No. of chickens	Lesion scores						Mean
		0	**1 +**	**2 +**	**3 +**	**4 +**	**5 +**	
Vehicle-only controls	10	1	3	4	1	1	0	1.55
Culture supernatant*	10	8	1	1	0	0	0	0.4
Alpha-toxoid	12	3	4	3	1	1	0	1.41
HP*	12	10	2	0	0	0	0	0.16
GPD*	10	7	2	1	0	0	0	0.4
tPFOR*	10	4	5	1	0	0	0	0.7
FBA*	10	4	6	0	0	0	0	0.6

487

488 * Immunized groups that had significantly fewer chickens with lesions compared to

489 unimmunized vehicle-only controls; Fisher's exact test, $p \leq 0.05$.

490

491

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ACCEPTED

494 Table 4. Intestinal lesion scores of birds immunized with two injections intramuscularly,

495 then infected with a moderate challenge by *C. perfringens*

Protein	No. of chickens	Lesion scores						Mean
		0	1+	2+	3+	4+	5+	
Vehicle-only controls	18	1	4	9	2	1	1	2.05
Mock controls	10	0	1	6	3	0	0	2.20
HP*	17	10	3	4	0	0	0	0.64
GPD	17	3	8	6	0	0	0	1.17
tPFOR	18	7	4	3	3	0	1	1.33
FBA	18	2	7	6	2	0	1	1.66

496 *Immunized group had significantly fewer chickens with lesions compared to

497 unimmunized vehicle-only controls; Fisher's exact test, $p \leq 0.05$.

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504 Table 5. Intestinal lesion scores of birds immunized with three injections intramuscularly,

505 then infected with a severe challenge by *C. perfringens*.

Protein	No. of chickens	Lesion scores						Mean
		0	**1+**	**2+**	**3+**	**4+**	**5+**	
Vehicle-only controls	22	0	5	5	6	4	2	2.68
Alpha toxoid/toxin* [a]	19	10	8	1	0	0	0	0.53
HP*	20	8	6	4	2	0	0	1.0
GPD	18	4	4	6	1	1	1	1.64
tPFOR*	19	9	2	6	2	0	0	1.05
GPD + HP	19	5	5	7	1	1	0	1.36

506 *Immunized groups that have significantly fewer chickens with lesions compared to

507 unimmunized vehicle-only controls; Fisher's exact test, $p \leq 0.05$.

508 [a] Birds in this group received alpha-toxoid in the first two injection and toxin in the third.

509

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ACCEPTED

512 Table 6. Intestinal lesion scores of birds immunized with three injections intramuscularly,

513 then infected with mild-moderate or severe challenge by *C. perfringens*

Protein	**No. of chickens**	**Lesion scores**						**Mean**
		0	**1 +**	**2 +**	**3 +**	**4 +**	**5 +**	
Groups infected with mild-moderate challenge								
Vehicle-only controls	10	0	5	2	0	1	2	2.3
FBA*	13	4	6	1	1	0	1	1.23
Groups infected with severe challenge								
Vehicle-only controls	11	0	3	2	3	1	2	2.72
Alpha-toxin[a]	10	0	2	4	4	0	0	2.2
FBA	14	1	4	9	0	0	0	1.57

514

515 *Immunized group that had significantly fewer chickens with lesions compared to

516 unimmunized vehicle-only controls; Fisher's exact test, $p \leq 0.05$.

517 [a]- Birds in this group received three injections of alpha-toxin where in the first and the

518 third injections were with 20 μg and the second was reduced to 10 μg.

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519 **Figure Legends**

520 **Figure 1. Recombinant *Clostridium perfringens* histidine-tagged proteins purified**
521 **from *Escherichia coli* cells.** (A) Coomassie stained purified proteins (B) Reactivity of
522 purified proteins to immune serum from chickens immune to necrotic enteritis. In each
523 panel, Lane 1- Alpha-toxin (45 kDa); Lane 2- Glyceraldehyde 3-phosphate
524 dehydrogenase (40 kDa); Lane 3- Fructose 1,6-biphosphate aldolase (35 kDa); Lane 4-
525 Truncated pyruvate: ferredoxin oxidoreductase (67 kDa); Lane 5- Hypothetical Protein
526 (90-100 kDa); Lane M- Molecular mass standards.

527

528 **Figure 2. Summary of mean lesion scores of birds from all immunized groups across**
529 **different experiments, together with the concurrent unimmunized controls.** VC-
530 vehicle-only controls, A- tox- alpha-toxin; FBA- Fructose 1,6-biphosphate aldolase;
531 GPD- Glyceraldehyde 3-phosphate dehydrogenase; tPFOR- Truncated pyruvate:
532 ferredoxin oxidoreductase; HP- Hypothetical protein; Sup- culture supernatant of *C.*
533 *perfringens*; G+H- combination of GPD and HP; Exp- Experiment. $^{+}$ -Birds in this group
534 were challenged for 3 days and necropsied on day- 6. $^{++}$ -Birds in this group were given a
535 severe challenge, like in Exp- 3. * -Immunized group that had significantly fewer
536 chickens with lesions compared to unimmunized vehicle-only controls; Fisher's exact
537 test, $p \leq 0.05$.

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ACCEPTED

540 **Figure 3. Serum IgY ELISA titers of broiler chickens immunized intramuscularly**
541 **with *Clostridium perfringens* purified proteins.** Serum collected at three time-points;
542 Day 0- Pre-immunization titer, Day 10- Mid-experiment, Day 20- Pre-challenge titer.
543 FBA- Fructose 1,6-biphosphate aldolase; GPD- Glyceraldehyde 3-phosphate
544 dehydrogenase; tPFOR- Truncated pyruvate: ferredoxin oxidoreductase; HP-
545 Hypothetical protein; Exp- Experiment. Exp.4A- Birds in this group were challenged for
546 3 days and necropsied on day- 6. Exp.4B- Birds in this group were given a severe
547 challenge, like in Exp- 3. * Significant titer values when compared to pre-immunization
548 titers, $p \leq 0.01$.

549

550 **Figure 4. Intestinal IgY and IgA ELISA titers of broiler chickens immunized**
551 **intramuscularly with *Clostridium perfringens* purified proteins.** Samples analyzed
552 were from pooled intestines collected from at least 10 chickens in each group. FBA-
553 Fructose 1,6-biphosphate aldolase; GPD- Glyceraldehyde 3-phosphate dehydrogenase;
554 tPFOR- Truncated pyruvate: ferredoxin oxidoreductase; HP- Hypothetical protein; Exp-
555 Experiment. Exp.4A- Birds in this group were challenged for 3 days and necropsied on
556 day- 6. Exp.4B- Birds in this group were given a severe challenge, like in Exp- 3.

557

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Fig. 1

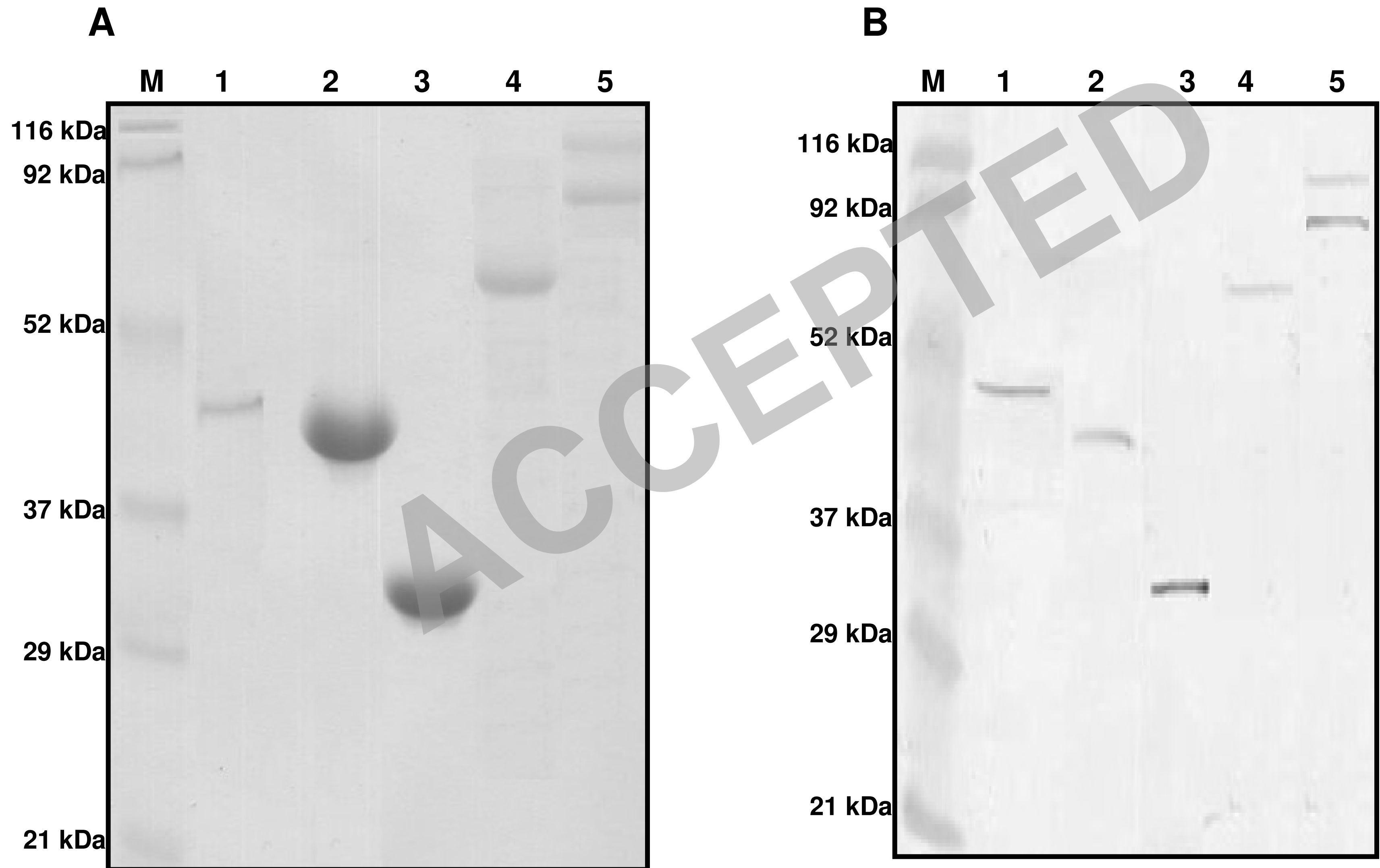

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Fig. 2

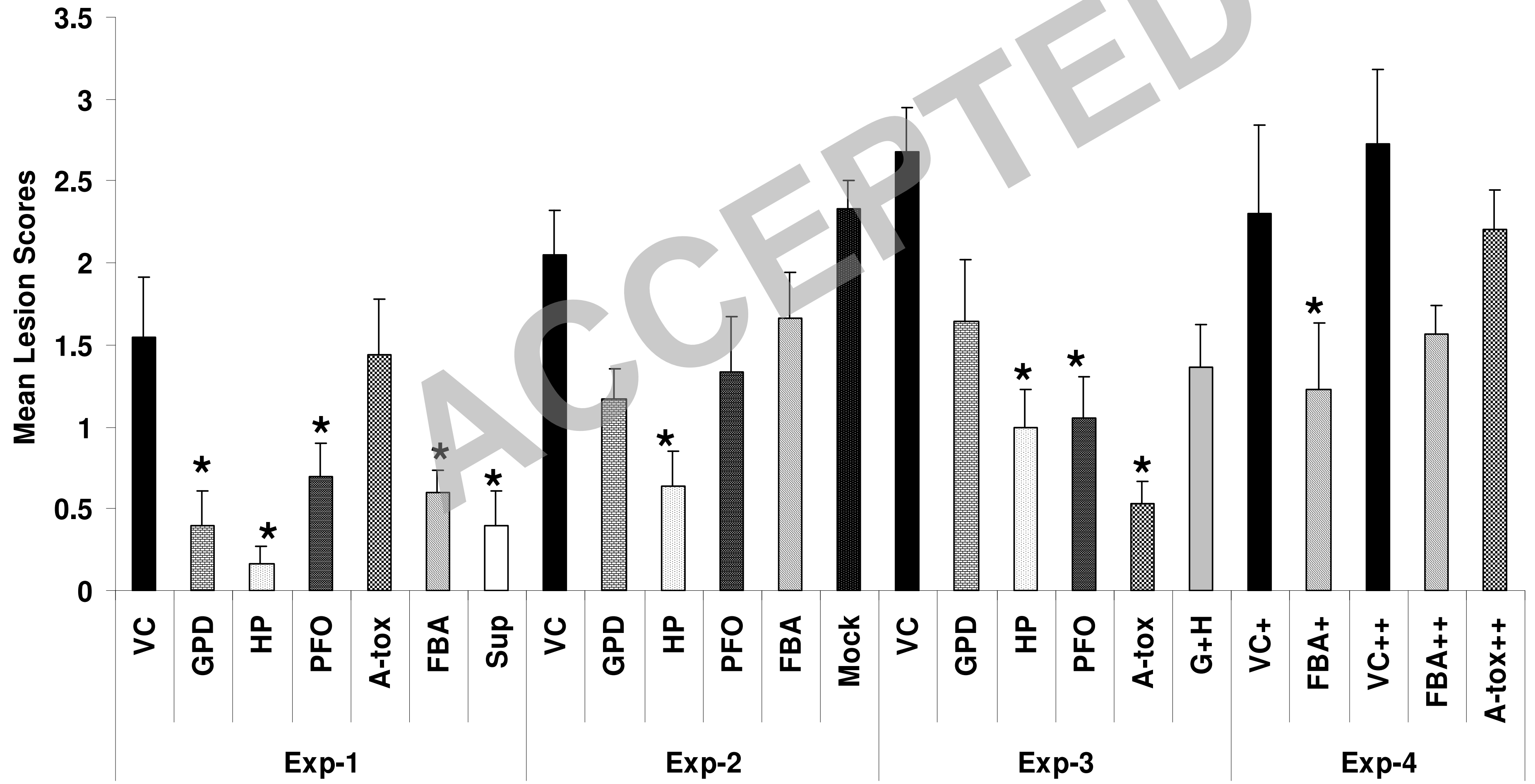

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Fig. 3

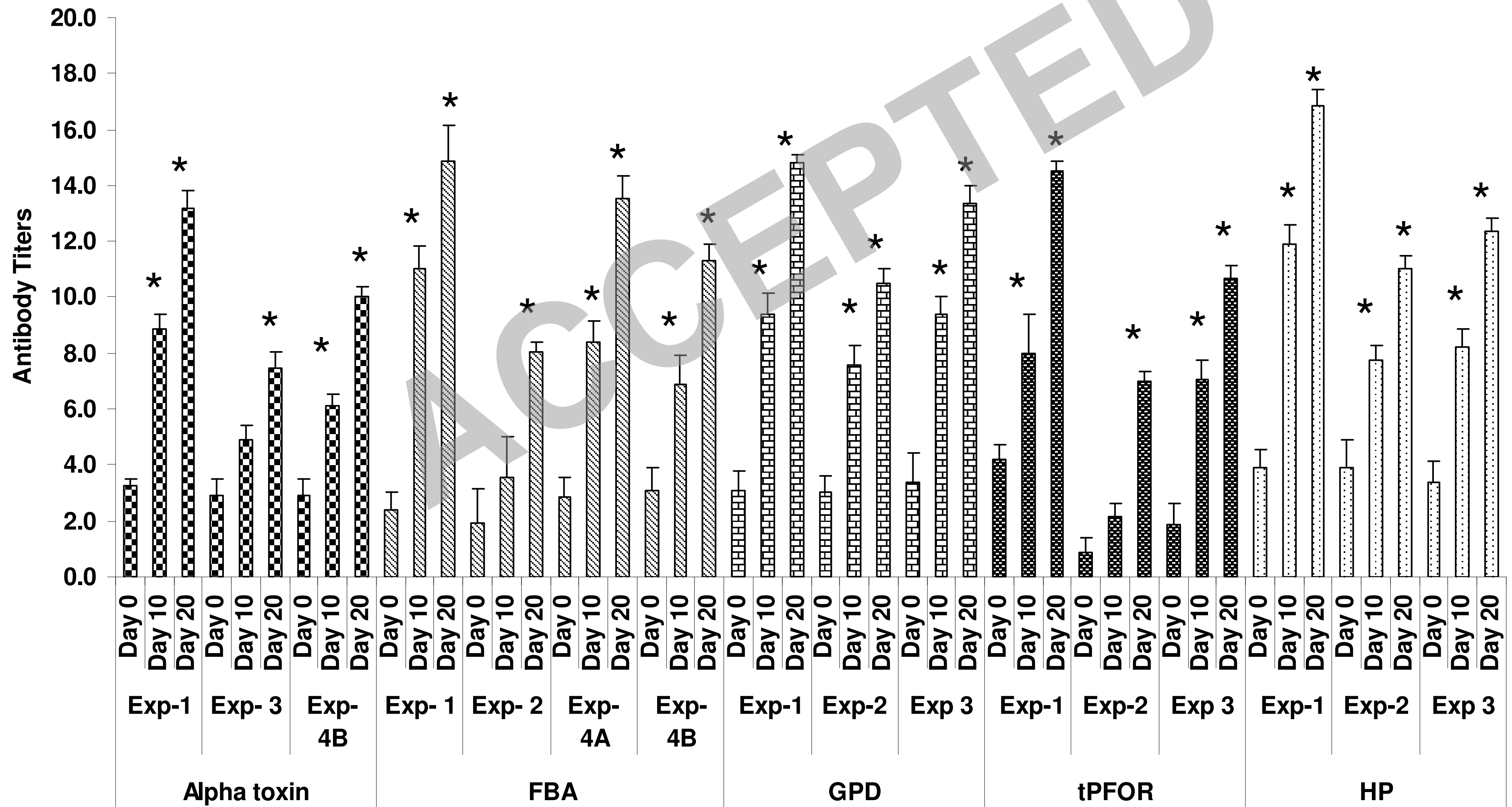

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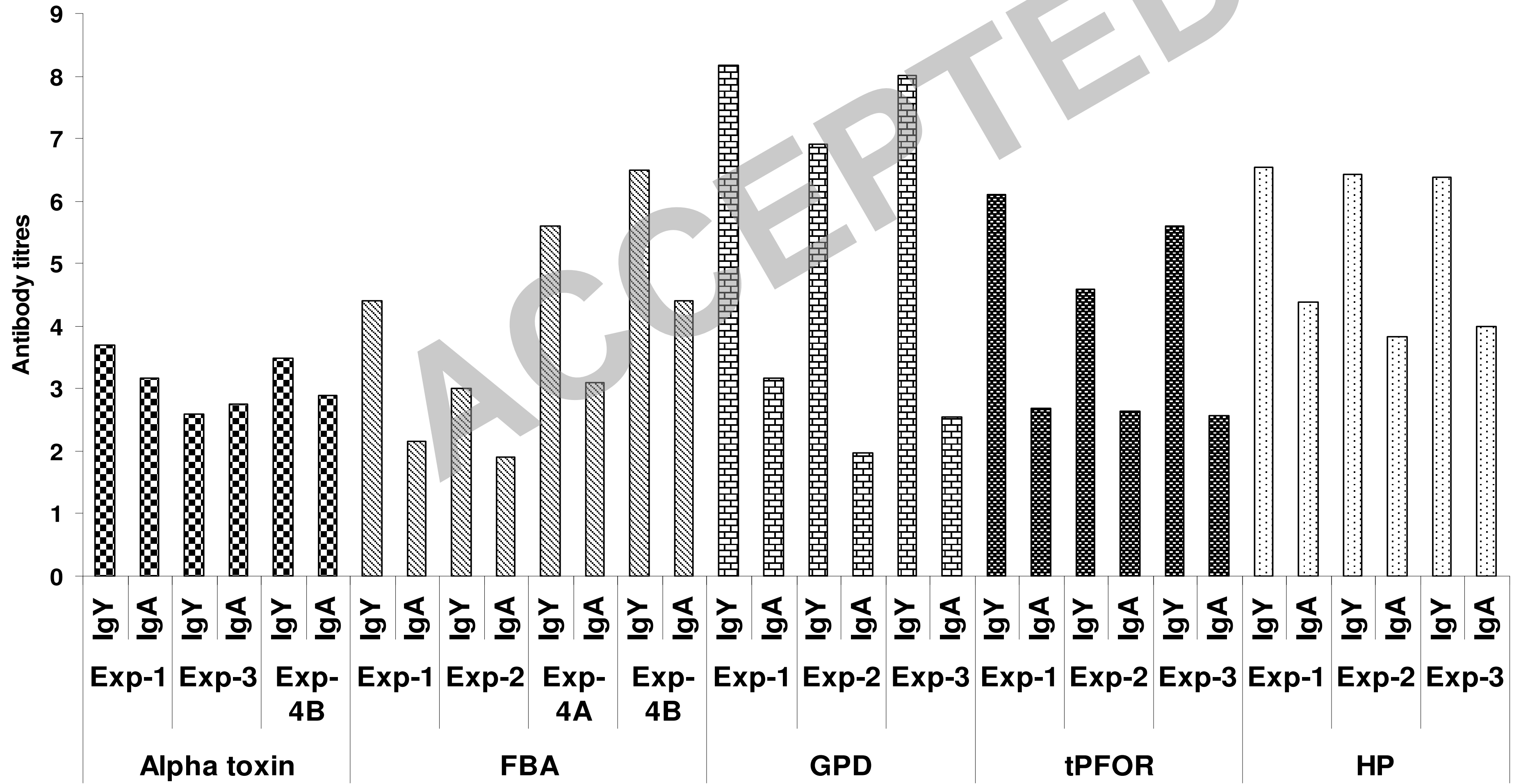
Fig. 4



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