Variable protection after vaccination of broiler chickens against necrotic enteritis using supernatants of different *Clostridium perfringens* strains

A. LANCKRIET, L. TIMBERMONT, V. EECKHAUT, F. HAESEBROUCK, R. DUCATELLE and F VAN IMMERSEEL*

Department of Pathology, Bacteriology and Avian Diseases, Research Group Veterinary Public Health and Zoonoses, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium

- Corresponding author. Mailing address : Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium. Phone: (0032) 09 264 77 48. Fax: (0032) 09 264 74 94. E-mail: filip.vanimmerseel@ugent.be

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Abstract

Necrotic enteritis is an economically important disease of chickens caused by *Clostridium perfringens*. Immunity to necrotic enteritis is not fully characterized yet, but previous reports indicate that immunoprotective potential is present in the secreted component of *C. perfringens.* This study aimed to compare the vaccine potential of the supernatants of 8 chicken strains of *C. perfringens* differing in origin, level of alpha toxin production and presence of *netB* gene. The supernatant of only one strain provided full protection, while one other strain provided partial protection against a severe infection challenge. Our results indicate that the protective characteristics of the supernatants are not solely based on the presence of NetB or alpha toxin.

1. Introduction

Clostridium perfringens is the causative agent of necrotic enteritis, an intestinal disease that affects industrial poultry worldwide [1, 2]. After the ban of growth promoting antibiotics in the European Union, necrotic enteritis has become much more widespread. It is mainly contained by using curative antibiotics or ionophore anticoccidials [3, 4]. The use of curative antibiotics and anticoccidials, however, holds the risk of inducing resistance among the *C. perfringens* population and the rest of the intestinal microbiota.

Vaccination would be a valuable approach for the prevention of necrotic enteritis. *C. perfringens* strains are ubiquitous and notorious for the wide range of toxins and

65 virulence factors they excrete in their environment [5]. These different virulence factors
66 and toxins may be considered as potential candidate antigens.
67 Previously, results from our research group suggested the presence of host-specific
68 virulence factors present in *C. perfringens* chicken strains isolated from necrotic enteritis
69 outbreaks [6]. The majority of the chicken strains are toxinotype A, meaning that they
70 carry the *plc* gene encoding alpha toxin [7-10]. For a long time, it was believed that this
71 alpha toxin, a metalloenzyme with lecithinase and sphingomyelinase activities, was the
72 major virulence factor involved in necrotic enteritis [11]. Recently, a novel pore forming
73 toxin, NetB, was discovered [12]. Whereas a *plc* deletion mutant from a virulent *C.*
74 *perfringens* chicken strain was still capable of inducing necrotic lesions in the gut of
75 experimentally infected broilers, a *netB* deletion mutant from the same strain was not [12,
76 13]. Although not involved in the onset of necrotic lesions, alpha toxin may still play a
77 role in the pathogenesis of the disease. The critical importance of NetB for the
78 development of necrotic enteritis is still under discussion as occasionally isolates that
79 lack the *netB* gene can be found .in birds suffering from necrotic enteritis and necrotic
80 enteritis has been reproduced with *netB* negative isolates.[2, 14-16]. Besides alpha toxin
81 and NetB, *C. perfringens* secretes growth inhibiting factors and hydrolytic proteins, such
82 as collagenases, that play or might play a role in the pathogenesis of necrotic enteritis [2,
83 17, 18]. However, the role of these proteins in immunity to necrotic enteritis in chickens
84 still needs to be elucidated. As the supernatant of *C. perfringens* contains a lot of
85 potential immunoreactive compounds, the supernatant and its content has been studied as
86 a potential vaccine to prevent clinical and subclinical necrotic enteritis with moderate
87 degrees of success [19, 20]. Up till now, no full immunity to necrotic enteritis has been

88 achieved with supernatant-based vaccines. Moreover, it is not known whether chicken

89 derived *C. perfringens* strains produce supernatants that differ in their capacity to induce

90 a strongly protective immune response.

91

92 The aim of this study was to compare the immunoprotective potential of the

93 supernatants from 8 *C. perfringens* strains with different characteristics with regard to

94 flock health status, NetB toxin and level of alpha toxin production.

95

96 **2. Materials and methods**

97 *2.1 Strains*

98 Eight *C. perfringens* type A strains isolated from chickens and belonging to different
99 genotypes, as analyzed by Pulsed Field Gel Electrophoresis (PFGE), were included [8].
100 All strains were used for supernatant production. All *C. perfringens* bacteria were grown
101 anaerobically at 37°C in brain heart infusion (BHI) broth (Oxoid, Basingstoke, UK). *C.*
102 *perfringens* strains 56 and 61 were used as challenge strains in an *in vivo* necrotic enteritis
103 model described below and have been shown to be highly virulent in the applied *in vivo*
104 model before [6, 21]. A summary of the characteristics of the strains is given in Table 1.

105

106 *2.2 Alpha toxin production and detection of the netB gene*

107 The alpha toxin production levels of the strains are shown in Table 1 and were
108 determined in an earlier report [8].

109

110 The presence of the *netB* gene was determined by PCR using the primers AKP78 (5′-
111 GCTGGTGCTGGAATAAATGC-3′) and AKP79 (5′-TCGCCATTGAGTAGTTTCCC-
112 3′), as described by Keyburn *et al.* [12]. Lactate dehydrogenase cytotoxicity assays were
113 performed as an indicator for NetB expression [12]. Overnight cultures were grown in
114 BHI. The supernatants were filter sterilized and dialyzed overnight against 10 mM Tris-
115 HCl pH 8.5. To test for cytotoxicity, the culture supernatants were added to the medium
116 of chicken hepatoma (LMH) cells (ATCC CRL-2117) in a 1:4 dilution with cell medium.
117 The cells were incubated for 3 hours at 37°C and 5% CO_2. Lactate dehydrogenase release
118 in the supernatant was used as an indicator of cytolysis and hence NetB production and

119 was measured using the Cytotoxicity Detection Kit (Roche Applied Sciences, Penzberg,
120 Germany). As a positive control, 10% Triton X-100 (Sigma Aldrich, St. Louis MO, USA)
121 was added to the cells. The negative control consisted of BHI 1:4 diluted with cell
122 medium that was added to the cells. Percentage cytotoxicity was determined relative to
123 the control groups. Experiments were repeated four times. For the LDH cytotoxicity
124 assays, a one-way analysis of variance was used to detect significant differences in the
125 relative cytotoxicity percentages of the supernatants ($P<0.05$) followed by a post-hoc
126 Bonferroni test for multiple comparisons ($P<0.0625$).
127
128 *2.3 Vaccines*
129 In trials 1 and 2, the *C. perfringens* strains were grown in BHI supplemented with
130 0.37% glucose for 14 hours at 37°C in an anaerobic (84 % N_2, 8 % CO_2 and 8 % H_2)
131 workstation (Ruskinn Technology, South Wales, UK). Supernatants derived from these
132 overnight cultures were concentrated by dialysis against a 20 kDa polyethyleneglycol
133 (PEG, Sigma Aldrich, St. Louis MO, USA) solution, followed by further concentration
134 and desalting using Centricon columns (Millipore, Billerica, MA, USA). Protein
135 concentrations from the supernatants were determined by the Bradford method with a
136 commercially available Bradford reagens (Bio-Rad, Hercules, CA, USA). The
137 concentrated supernatant samples were diluted in PBS to a final concentration of
138 7 µg/200 µl or 70 µg/200 µl respectively.
139
140 *2.4 Experimental setup of the in vivo trials*
141 The *in vivo* necrotic enteritis challenge model was applied as described previously

142 [21]. Groups of 30 (trial 1) or 27 (trial 2 and 3) broilers were fed a wheat/rye-based
143 (43%/7.5%) diet, with soybean meal as protein source.
144 In trial 1, vaccination took place in 8 groups of birds with supernatants from 8 different
145 strains (strains 7, 11, 23, 37, 43, 48, 56, 61). On day 3 and day 12 post-hatching, the birds
146 were vaccinated subcutaneously in the neck with a 200 µl dose of supernatant containing
147 7 µg and 70 µg total protein respectively. QuilA (Brenntag Biosector, Frederikssund,
148 Denmark) was used as an adjuvant (50 µg/bird/vaccination). At the same time, one
149 control group got placebo vaccinated with PBS and 50µg of QuilA/bird/vaccination.
150 Another control group was left unvaccinated. In trial 2, only the supernatant of strain 23
151 was used for vaccination in the same way as described for trial 1. The control group was
152 vaccinated with PBS and QuilA. In trial 3, no prior vaccination took place.
153 In all three trials, Nobilis Gumboro D78 vaccine (Schering-Plough Animal Health,
154 Brussels, Belgium) was given in the drinking water on day 16 in all groups. From day 17
155 onwards, the same diet was used with the exception that fishmeal (30%) replaced soy
156 bean as protein source. On days 17, 18, 19 and 20, oral challenge was performed three
157 times a day with approximately 4.10^8 cfu *C. perfringens* bacteria. On day 18, all birds
158 were orally inoculated with a ten-fold dose of Paracox-5 (Schering-Plough Animal
159 Health).In trial 1, oral challenge was performed with virulent strain 56 whereas in trial 2,
160 the chickens were challenged with virulent strain 56 or 61. In trial 3, the virulence of
161 strain 23 was compared to that of strain 56 in the necrotic enteritis challenge model. In
162 trial 3, a negative control group was included consisting of birds to which only a 10-fold
163 dose of Paracox-5 was administered.
164 On days 22, 23 and 24, each time one third of the birds in each group were

165 euthanized and necropsied. Intestinal lesions in the small intestine (duodenum to ileum)
166 were scored as described by Keyburn *et al.*[13]. Birds with lesion scores of 2 (1 to 5
167 necrotic lesions) or more were classified as necrotic enteritis positive. The data were
168 analyzed with SPSS Statistics 17.0 software (SPSS Inc., Chicago) using the binary
169 logistic regression method to compare the number of necrotic enteritis positive animals
170 within the test groups. Bonferroni's correction for multiple comparisons was applied
171 ($P<0.05/n$) for the first vaccination trial and significance was determined at $P < 0.005$.
172 For all other trials, significance was determined at $P<0.05$.
173

174 **3. Results**

175 *3.1 Detection of the netB gene and NetB cytotoxic effect towards LMH cells*

176 PCR showed that strains 7, 11, 43 and 48 were *netB* negative, whereas strains 23, 37,
177 56 and 61 carry the *netB* gene. The *in vitro* cytopathic effect of all supernatants towards
178 LMH cells was determined as an indicator for NetB activity [12, 15]. The relative
179 cytotoxicity percentages of the supernatants from the different strains are shown in Table
180 1. The post-hoc Bonferroni method for multiple comparison confirmed that the
181 supernatants of all but one (strain 11) *netB* negative strains were significantly less
182 cytotoxic towards LMH cells than the supernatants of *netB* positive isolates 23, 37, 56
183 and 61 ($P<0.00625$).
184

185 *3.2 Vaccination experiments*

186 The vaccination experiment (trial 1) clearly showed that vaccination with the
187 supernatant of *netB* negative strains 7, 11 and 43 did not result in immunoprotection

188 (Table 2). Vaccination with supernatant of *netB* positive strains 37 and 56 did not result
189 in immunoprotection either. Vaccination with supernatant from strains 23 and 48 resulted
190 in a significant decrease in number of birds with necrotic lesions ($P<0.005$). Only
191 vaccination with supernatant of strain 23, a *netB* positive strain and low alpha toxin
192 producer, totally prevented the development of necrotic lesions after severe challenge.
193 The supernatant of strain 48 (*netB* negative, high alpha toxin producer) provided partial
194 protection.

195 Since the toxins in the supernatants were not neutralized, a variable number of chicks
196 died shortly after vaccination. High percentages of dead chicks were observed after
197 vaccination with supernatant of strains 11 and 48 (high alpha toxin producers) and strain
198 61 (low alpha toxin producer).

199 In a second vaccination trial, none of the chickens that received subcutaneous
200 vaccination with supernatant of strain 23 showed lesions, while 20% and 19% of the
201 chickens vaccinated with adjuvant only were positive for necrotic lesions after challenge
202 with virulent strain 56 and 61 respectively (Table 2).
203
204
205

206 **4. Discussion**

207 Only vaccination with two supernatants out of eight, i.e. those from strains 23 and
208 48, significantly protected the birds against necrotic enteritis (trial 1). Vaccination with
209 supernatant of strain 23 resulted in full protection against necrotic enteritis after
210 challenge with two different virulent strains (trial 2). To our knowledge, this is the first
211 time that vaccination with supernatant has such a profound effect. Previous reports on
212 vaccination experiments with supernatants or toxoids show a significant reduction in the
213 number of animals with necrotic lesions or partial protection but not total protection [19].
214 Vaccination of parent birds with type A or C toxoid decreased the occurrence of enteritis
215 or hepatitis lesions with 5 to 15% in the progeny but did not totally prevent the
216 development of necrotic enteritis [22].

217

218 When the immunoprotective potential of the eight supernatants is compared with the
219 presence of the *netB* gene or level of presumed NetB-associated hepatocyte toxicity of
220 the respective strains, it shows that the supernatants of 3 out of 4 *netB* negative strains
221 confer no immunoprotective potential at all. Presence of a *netB* gene in combination with
222 a cytopathic effect of the supernatants towards LMH cells was not associated with
223 immunoprotective potential for the supernatants of strains 37, 56 and 61. All four *netB*
224 positive strains, 23 37, 56 and 61, are characterised by high relative cytotoxicity
225 percentages towards LMH cells of over 75%. For the Australian virulent necrotic
226 enteritis strain EHE-NE18, it was confirmed that deletion of the *netB* gene resulted in
227 loss of cytotoxicity [12]. In this study, two *netB* negative strains, 11 and 48, show
228 medium relative cytotoxicity percentages towards LMH cells of 61% and 43%

229 respectively. These results indicate that for some strains, other proteins may be involved
230 in the cytotoxicity towards LMH cells. Caution is thus recommended when correlating
231 LDH cytotoxicity assays with levels of NetB expression.
232 Since strain 23 produces low levels of alpha toxin *in vitro* whereas strain 48 produces
233 high levels, the immunoprotective potential of the supernatans does not seem to depend
234 on he amount of alpha toxin present. Remarkably, many chicks died shortly after
235 vaccination with supernatants of the high alpha toxin producers strains 11 and 48,
236 indicating that alpha toxin could play a role in the high rate of mortality often observed
237 in severe cases of clinical necrotic enteritis. This shock effect has been described before
238 [11, 19]. It has been shown that a *plc* mutant is still capable of inducing necrotic lesions
239 [13]. This finding does not rule out that alpha toxin can play a secondary role in necrotic
240 enteritis. When the intestinal epithelium is severely compromised, alpha toxin can reach
241 the blood stream resulting in damage to organs or blood vessels. The supernatant of
242 strain 61, a low alpha toxin producer, might be toxic to the chicks due to another
243 unknown combination of toxic compounds.
244
245 The results of this study show that the immunity to necrotic enteritis induced after
246 immunization with supernatant of *C. perfringens* strains is not entirely determined by the
247 concentration of alpha toxin or NetB but also involves other immunogens. These findings
248 explain the varying degrees of partial protection observed after immunizations with
249 vaccines based on a single antigenic protein such as alpha toxin, a hypothetical protein
250 (presumably a metalloprotease) and pyruvate-ferredoxine oxidoreductase,.. where
251 necrotic enteritis still developed in the flock after experimental challenge but the total

252 number of birds with lesions was significantly reduced [19, 23]. Which unique antigenic
253 features causes the supernatant of strain 23 to protect broilers against the development of
254 necrotic enteritis even after severe challenge with different virulent strains is currently
255 not known.
256
257 In conclusion, our results indicate that the immunoprotective potential of the
258 supernatants of *C. perfringens* necrotic enteritis strains is not solely determined by the
259 strains' level of alpha toxin expression or by the presence of the *netB* gene. The secreted
260 component of strain 23 owes its immunoprotective features to a so far unknown
261 combination of immunogenic compounds.
262
263

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265

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268

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336
337

338 **TABLES**

339 **Table 1.** Characteristics of the strains and results of the lactate dehydrogenase

340 cytotoxicity assay of LMH cells treated with ther1:4 diluted supernatants of these strains

Strain	**Health status of the flock[1]**	**Alpha toxin production[2]**	**Detection of the *netB* gene**	**Relative percentage cytotoxicity of the supernatant[3]**	**Standard deviation**
7	healthy	Low	-	1	2
11	healthy	high	-	61	10
23	healthy	Low	+	75	15
37	diseased	moderate	+	86	17
43	diseased	moderate	-	13.	4
48	diseased	high	-	43	18
56	diseased	moderate	+	87.	12
61	diseased	Low	+	89.	15

341 [1] Diseased means that there was a necrotic enteritis outbreak in the flock.

342 [2] As determined in [8].

343 [3]Percentage cytotoxicity of the supernatants were determined relative to control wells

344 treated with 10% Triton X-100 (positive control) or 1:4 diluted BHI (negative control).

345 The values are averages of 4 independent trials.

346

347 **Table 2.** Total number of birds with macroscopic necrotic enteritis lesions (lesion score
348 ≥2) in the vaccination experiments

Trial	Vaccination Group*	Strain used for challenge	Number of animals with lesions/total number	Percentage of animal with lesions	Number of dead chicks due to vaccination/total number§
1	SN 7	56	20/29	69 %	0/30
	SN 11	56	8/13	61 %	16/30
	SN 23	56	0/29[a]	0 %	0/30
	SN 37	56	13/24	54 %	2/30
	SN 43	56	23/30	76 %	0/30
	SN 48	56	3/17[a]	17 %	13/30
	SN 56	56	12/24	50 %	2/30
	SN 61	56	4/12	33 %	18/30
	PBS	56	12/25	47 %	0/30
	no vaccination	56	15/26	56 %	0/30
2	PBS	56	5/25	20 %	0/27
	SN 23	56	0/25[b]	0 %	0/27
	PBS	61	5/26	19 %	0/27
	SN 23	61	0/25[c]	0 %	0/27

349 *SN= supernatant

350 §only chicks that died within 24 hours after vaccination are mentioned

351 [a] values with superscripts differ significantly (P<0.005 for trial 1) from both the PBS-vaccinated
352 as the non-vaccinated group (trial 1)

353 [b,c] Values with superscripts differ significantly (P<0.05) from the control group vaccinated with
354 PBS group and infected with the respective strain (trial 2)

355