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

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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
virulence factors they excrete in their environment [5]. These different virulence factors
and toxins may be considered as potential candidate antigens.

Previously, results from our research group suggested the presence of host-specific
virulence factors present in *C. perfringens* chicken strains isolated from necrotic enteritis
outbreaks [6]. The majority of the chicken strains are toxinotype A, meaning that they
carry the *plc* gene encoding alpha toxin [7-10]. For a long time, it was believed that this
alpha toxin, a metalloenzyme with lecithinase and sphingomyelinase activities, was the
major virulence factor involved in necrotic enteritis [11]. Recently, a novel pore forming
toxin, NetB, was discovered [12]. Whereas a *plc* deletion mutant from a virulent *C.
perfringens* chicken strain was still capable of inducing necrotic lesions in the gut of
experimentally infected broilers, a *netB* deletion mutant from the same strain was not [12,
13]. Although not involved in the onset of necrotic lesions, alpha toxin may still play a
role in the pathogenesis of the disease. The critical importance of NetB for the
development of necrotic enteritis is still under discussion as occasionally isolates that
lack the *netB* gene can be found. in birds suffering from necrotic enteritis and necrotic
enteritis has been reproduced with *netB* negative isolates.[2, 14-16]. Besides alpha toxin
and NetB, *C. perfringens* secretes growth inhibiting factors and hydrolytic proteins, such
as collagenases, that play or might play a role in the pathogenesis of necrotic enteritis [2,
17, 18]. However, the role of these proteins in immunity to necrotic enteritis in chickens
still needs to be elucidated. As the supernatant of *C. perfringens* contains a lot of
potential immunoreactive compounds, the supernatant and its content has been studied as
a potential vaccine to prevent clinical and subclinical necrotic enteritis with moderate
degrees of success [19, 20]. Up till now, no full immunity to necrotic enteritis has been
achieved with supernatant-based vaccines. Moreover, it is not known whether chicken
derived *C. perfringens* strains produce supernatants that differ in their capacity to induce
a strongly protective immune response.

The aim of this study was to compare the immunoprotective potential of the
supernatants from 8 *C. perfringens* strains with different characteristics with regard to
flock health status, NetB toxin and level of alpha toxin production.
2. Materials and methods

2.1 Strains

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

2.2 Alpha toxin production and detection of the netB gene

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

The presence of the *netB* gene was determined by PCR using the primers AKP78 (5′-GCTGGTGCTGGAATAATGC-3′) and AKP79 (5′-TCGCCATTGAGTAGTTTCCC-3′), as described by Keyburn et al. [12]. Lactate dehydrogenase cytotoxicity assays were performed as an indicator for NetB expression [12]. Overnight cultures were grown in BHI. The supernatants were filter sterilized and dialyzed overnight against 10 mM Tris-HCl pH 8.5. To test for cytotoxicity, the culture supernatants were added to the medium of chicken hepatoma (LMH) cells (ATCC CRL-2117) in a 1:4 dilution with cell medium. The cells were incubated for 3 hours at 37°C and 5% CO₂. Lactate dehydrogenase release in the supernatant was used as an indicator of cytolysis and hence NetB production and
was measured using the Cytotoxicity Detection Kit (Roche Applied Sciences, Penzberg, Germany). As a positive control, 10% Triton X-100 (Sigma Aldrich, St. Louis MO, USA) was added to the cells. The negative control consisted of BHI 1:4 diluted with cell medium that was added to the cells. Percentage cytotoxicity was determined relative to the control groups. Experiments were repeated four times. For the LDH cytotoxicity assays, a one-way analysis of variance was used to detect significant differences in the relative cytotoxicity percentages of the supernatants (P<0.05) followed by a post-hoc Bonferroni test for multiple comparisons (P<0.0625).

2.3 Vaccines

In trials 1 and 2, the C. perfringens strains were grown in BHI supplemented with 0.37% glucose for 14 hours at 37°C in an anaerobic (84 % N2, 8 % CO2 and 8 % H2) workstation (Ruskinn Technology, South Wales, UK). Supernatants derived from these overnight cultures were concentrated by dialysis against a 20 kDa polyethyleneglycol (PEG, Sigma Aldrich, St. Louis MO, USA) solution, followed by further concentration and desalting using Centricon columns (Millipore, Billerica, MA, USA). Protein concentrations from the supernatants were determined by the Bradford method with a commercially available Bradford reagens (Bio-Rad, Hercules, CA, USA). The concentrated supernatant samples were diluted in PBS to a final concentration of 7 µg/200 µl or 70 µg/200 µl respectively.

2.4 Experimental setup of the in vivo trials

The in vivo necrotic enteritis challenge model was applied as described previously
Groups of 30 (trial 1) or 27 (trial 2 and 3) broilers were fed a wheat/rye-based diet, with soybean meal as protein source. In trial 1, vaccination took place in 8 groups of birds with supernatants from 8 different strains (strains 7, 11, 23, 37, 43, 48, 56, 61). On day 3 and day 12 post-hatching, the birds were vaccinated subcutaneously in the neck with a 200 µl dose of supernatant containing 7 µg and 70 µg total protein respectively. QuilA (Brenntag Biosector, Frederikssund, Denmark) was used as an adjuvant (50 µg/bird/vaccination). At the same time, one control group got placebo vaccinated with PBS and 50µg of QuilA/bird/vaccination. Another control group was left unvaccinated. In trial 2, only the supernatant of strain 23 was used for vaccination in the same way as described for trial 1. The control group was vaccinated with PBS and QuilA. In trial 3, no prior vaccination took place.

In all three trials, Nobilis Gumboro D78 vaccine (Schering-Plough Animal Health, Brussels, Belgium) was given in the drinking water on day 16 in all groups. From day 17 onwards, the same diet was used with the exception that fishmeal (30%) replaced soy bean as protein source. On days 17, 18, 19 and 20, oral challenge was performed three times a day with approximately $4.10^8$ cfu *C. perfringens* bacteria. On day 18, all birds were orally inoculated with a ten-fold dose of Paracox-5 (Schering-Plough Animal Health). In trial 1, oral challenge was performed with virulent strain 56 whereas in trial 2, the chickens were challenged with virulent strain 56 or 61. In trial 3, the virulence of strain 23 was compared to that of strain 56 in the necrotic enteritis challenge model. In trial 3, a negative control group was included consisting of birds to which only a 10-fold dose of Paracox-5 was administered.

On days 22, 23 and 24, each time one third of the birds in each group were
euthanized and necropsied. Intestinal lesions in the small intestine (duodenum to ileum) were scored as described by Keyburn et al.[13]. Birds with lesion scores of 2 (1 to 5 necrotic lesions) or more were classified as necrotic enteritis positive. The data were analyzed with SPSS Statistics 17.0 software (SPSS Inc., Chicago) using the binary logistic regression method to compare the number of necrotic enteritis positive animals within the test groups. Bonferroni’s correction for multiple comparisons was applied (P<0.05/n) for the first vaccination trial and significance was determined at P < 0.005. For all other trials, significance was determined at P<0.05.

3. Results

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

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

3.2 Vaccination experiments

The vaccination experiment (trial 1) clearly showed that vaccination with the supernatant of netB negative strains 7, 11 and 43 did not result in immunoprotection
Vaccination with supernatant of *netB* positive strains 37 and 56 did not result in immunoprotection either. Vaccination with supernatant from strains 23 and 48 resulted in a significant decrease in number of birds with necrotic lesions (P<0.005). Only vaccination with supernatant of strain 23, a *netB* positive strain and low alpha toxin producer, totally prevented the development of necrotic lesions after severe challenge. The supernatant of strain 48 (*netB* negative, high alpha toxin producer) provided partial protection.

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

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

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

When the immunoprotective potential of the eight supernatants is compared with the presence of the netB gene or level of presumed NetB-associated hepatocyte toxicity of the respective strains, it shows that the supernatants of 3 out of 4 netB negative strains confer no immunoprotective potential at all. Presence of a netB gene in combination with a cytopathic effect of the supernatants towards LMH cells was not associated with immunoprotective potential for the supernatants of strains 37, 56 and 61. All four netB positive strains, 23 37, 56 and 61, are characterised by high relative cytotoxicity percentages towards LMH cells of over 75%. For the Australian virulent necrotic enteritis strain EHE-NE18, it was confirmed that deletion of the netB gene resulted in loss of cytotoxicity [12]. In this study, two netB negative strains, 11 and 48, show medium relative cytotoxicity percentages towards LMH cells of 61% and 43%
respectively. These results indicate that for some strains, other proteins may be involved in the cytotoxicity towards LMH cells. Caution is thus recommended when correlating LDH cytotoxicity assays with levels of NetB expression.

Since strain 23 produces low levels of alpha toxin \textit{in vitro} whereas strain 48 produces high levels, the immunoprotective potential of the supernatants does not seem to depend on the amount of alpha toxin present. Remarkably, many chicks died shortly after vaccination with supernatants of the high alpha toxin producers strains 11 and 48, indicating that alpha toxin could play a role in the high rate of mortality often observed in severe cases of clinical necrotic enteritis. This shock effect has been described before \cite{11, 19}. It has been shown that a \textit{plc} mutant is still capable of inducing necrotic lesions \cite{13}. This finding does not rule out that alpha toxin can play a secondary role in necrotic enteritis. When the intestinal epithelium is severely compromised, alpha toxin can reach the blood stream resulting in damage to organs or blood vessels. The supernatant of strain 61, a low alpha toxin producer, might be toxic to the chicks due to another unknown combination of toxic compounds.

The results of this study show that the immunity to necrotic enteritis induced after immunization with supernatant of \textit{C. perfringens} strains is not entirely determined by the concentration of alpha toxin or NetB but also involves other immunogens. These findings explain the varying degrees of partial protection observed after immunizations with vaccines based on a single antigenic protein such as alpha toxin, a hypothetical protein (presumably a metalloprotease) and pyruvate-ferredoxine oxidoreductase, where necrotic enteritis still developed in the flock after experimental challenge but the total
number of birds with lesions was significantly reduced [19, 23]. Which unique antigenic
to the supernatant of strain 23 to protect broilers against the development of
necrotic enteritis even after severe challenge with different virulent strains is currently
not known.

In conclusion, our results indicate that the immunoprotective potential of the
supernatants of *C. perfringens* necrotic enteritis strains is not solely determined by the
strains’ level of alpha toxin expression or by the presence of the *netB* gene. The secreted
component of strain 23 owes its immunoprotective features to a so far unknown
combination of immunogenic compounds.
5. Acknowledgements

We would like to thank Renzo Vercammen for his skilful technical assistance. This work was supported by the Institute for Science and Technology, Flanders (IWT).
6. References


[8] Gholamiandekhordi AR, Ducatelle R, Heyndrickx M, Haesebrouck F, Van Immerseel F. Molecular and phenotypical characterization of *Clostridium perfringens*...


Table 1. Characteristics of the strains and results of the lactate dehydrogenase cytotoxicity assay of LMH cells treated with 1:4 diluted supernatants of these strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Health status of the flock(^1)</th>
<th>Alpha toxin production(^2)</th>
<th>Detection of the netB gene</th>
<th>Relative percentage cytotoxicity of the supernatant(^3)</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>healthy</td>
<td>Low</td>
<td>-</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>11</td>
<td>healthy</td>
<td>high</td>
<td>-</td>
<td>61</td>
<td>10</td>
</tr>
<tr>
<td>23</td>
<td>healthy</td>
<td>Low</td>
<td>+</td>
<td>75</td>
<td>15</td>
</tr>
<tr>
<td>37</td>
<td>diseased</td>
<td>moderate</td>
<td>+</td>
<td>86</td>
<td>17</td>
</tr>
<tr>
<td>43</td>
<td>diseased</td>
<td>moderate</td>
<td>-</td>
<td>13.</td>
<td>4</td>
</tr>
<tr>
<td>48</td>
<td>diseased</td>
<td>high</td>
<td>-</td>
<td>43</td>
<td>18</td>
</tr>
<tr>
<td>56</td>
<td>diseased</td>
<td>moderate</td>
<td>+</td>
<td>87.</td>
<td>12</td>
</tr>
<tr>
<td>61</td>
<td>diseased</td>
<td>Low</td>
<td>+</td>
<td>89.</td>
<td>15</td>
</tr>
</tbody>
</table>

\(^1\) Diseased means that there was a necrotic enteritis outbreak in the flock.

\(^2\) As determined in [8].

\(^3\) Percentage cytotoxicity of the supernatants were determined relative to control wells treated with 10% Triton X-100 (positive control) or 1:4 diluted BHI (negative control). The values are averages of 4 independent trials.
Table 2. Total number of birds with macroscopic necrotic enteritis lesions (lesion score $\geq 2$) in the vaccination experiments

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

*SN= supernatant  
§only chicks that died within 24 hours after vaccination are mentioned  
\(^a\) values with superscripts differ significantly (P<0.005 for trial 1) from both the PBS-vaccinated as the non-vaccinated group (trial 1)  
\(^b,c\) Values with superscripts differ significantly (P<0.05) from the control group vaccinated with PBS group and infected with the respective strain (trial 2)