An Experimental Reproduction of Necrotic Enteritis in Broiler Chickens

Keywords: Clostridium perfringens; Necrotic enteritis; Broiler; Experimental model

Abstract

Clostridium perfringens is the main causative agent of avian necrotic enteritis (NE), an enteric infectious disease considered among the most important diseases in the poultry industry. Currently, there are numerous reports of experimental reproduction of NE using different inoculation protocols along with various predisposing factors which produce highly variable results. These models represent a fraction of a wide range of farming conditions under which disease can develop. This work describes the experimental induction of C. perfringens NE in broiler chickens after a short feed withdrawal of 12 hours prior to bacterial challenge. Broiler chicks fed with commercial chick starter feed 14 days post-hatch were afterwards offered feed mixed with C. perfringens twice a day for three consecutive days. On average, over 60% of challenged birds developed typical gross lesions. The results show that it is possible to reproduce the disease under conditions similar to those found in poultry farms which are not covered by other developed models. This model proved to be effective in the experimental reproduction of NE, allowing the evaluation of pathological parameters.

Introduction

Clostridium perfringens is a Gram-positive, rod-shaped, anaerobic, spore-forming bacterium that is commonly found in soil, sewage and in the gastro-intestinal tract of animals and humans as a member of the normal gut microbiota. According to the current classification, C. perfringens isolates are divided into five types (A, B, C, D and E) on the basis of the production of four major toxins (alpha, beta, epsilon and iota) [1]. Certain strains of C. perfringens type A cause necrotic enteritis (NE) in poultry [2,3]. In broilers, NE appears as a sudden increase in mortality occurring at any time during the production cycle, up to 1% per day and it will continue for up to two weeks if the affected flock left untreated. The subclinical form of the disease is characterized by non-specific signs such as poor digestion, reduced weight gain and increased feed conversion ratio, without obvious increase in mortality. Typical necrotic lesions can be observed in the intestinal tract during necropsy [3,4]. Both presentations of the disease have become economically significant problems for the broiler industry worldwide.

NE is considered a complex and multi-factorial disease. Experimental attempts to reproduce NE have required the inclusion of one or more potential predisposing factors. Lesions of NE associated with the challenge of birds with C. perfringens alone have been reported in a relatively low proportion of animals [5]. Recently, an effective model of NE which reproduced consistently several clinical signs of the disease and intestinal lesions in birds fed with a high-protein diet has been described by Cooper and Songer [6]. Although high protein diet would serve as potential predisposing factor, the successful use of this model seems to depend on the complete preservation of virulent characteristics of the pathogenic strain used. Therefore, the goal of the present report was to determine if the use of an optimized culture of a freshly pathogenic C. perfringens strain can cause a consistent reproduction of the disease in broiler chicks fed with regular feed but starved for 12 hours as predisposing condition.

Materials and Methods

Chickens, facilities and experiment design

Cobb Broiler chicks were obtained as one-day old hatchlings from a commercial hatchery (Buenos Aires, Argentina). Birds were housed in biosafety level 2 facilities located in the Veterinary and Agriculture Research Center (CICVyA-INTA), with controlled temperature and humidity and automated ventilation system. Eighty chicks were divided into 4 groups, 20 birds per group. Each group was housed in galvanized feed trays (regular diet). Commercial feed was mixed with fishmeal in a 50:50 proportion to obtain a high protein diet (HPD) to reproduce conditions favoring C. perfringens lesions used in previous assays [6]. Birds used in these studies were unvaccinated and fed with ration free of antimicrobial growth promoters.

Clostridium perfringens strain and inoculum preparation

The C. perfringens strain used in these studies was isolated from the intestinal lesions of a three weeks old broiler chicken during...
an outbreak of NE in a commercial poultry farm of Buenos Aires, Argentina in 2012. Molecular toxinotyping shows that this isolate was a type A strain negative for NetB [7] and cpb2 [6]. For inoculums preparation, a glycerol aliquot of the mentioned strain, obtained immediately after the first isolation, was streaked onto a blood agar plate with 5% defibrinated bovine blood. After incubation in anaerobic atmosphere (5% H2:5% CO2:90% N2) at 37 °C for 18 hours, 1-2 colonies were transferred into 10 ml cooked meat medium (CMM; Difco) and incubated in anaerobiosis at 37 °C for 12 hours. Then, this culture was inoculated into 100 ml of thioglycollate broth (FTG) and cultured as before. The mentioned FTG culture was diluted 1:10 in sterile CMM and incubated in anaerobiosis at 37 °C for 12 hours. One hundred ml of the last CMM culture was used to inoculate 1 L of FTG, and incubated during 12 hours. After incubation, a drop plate method [8] was used to assess the number of colony-forming units (CFU) in each dose of the inoculums. Final culture was mixed with feed in a 1:1 (w/v) ratio and administered immediately to birds. The procedure was repeated for each dose of inoculum used during the challenge (total challenge feeding, n=6).

Challenge protocol
A 2×2 arrangement of treatments was used to test the effect of C. perfringens challenge (unchallenged vs. challenged) and diet effect (regular vs. high protein). As mentioned above, four groups of 20 birds were assigned to each treatment. On day 15 and before first challenge birds were fasted for 12 hours. Between days 16 to 18, birds were challenged twice a day with final C. perfringens culture mixed with feed. Uneaten feed was discarded before each subsequent feeding. Sterile FTG mixed with feed was administered to a group of birds fed with regular diet and to a group of birds with HPD as negative control. This protocol was applied equally to birds fed with regular rations and high protein ration. This challenge was repeated three times and results were expressed as the mean of the three different trials.

Clinical and pathological examination
After first dose of inoculum, birds were observed three times a day for the prompt detection of clinical signs. On day 19 (three days after first challenge) birds were euthanized and necropsy was performed immediately for examination of gross lesions. Intestinal tracts were removed and lesions were scored blindly by two experienced pathologists (scores: 0=no apparent gross lesions; 1=removable fibrin deposit; 2=isolated focal necrosis or ulceration (1 to 5 foci); 3=multiple focal necrosis or ulceration (6 or more foci); 4=extensive areas of necrosis; 5=diffuse necrosis, presence of attached pseudomembrane). To confirm the identity of intestinal lesions, tissue samples were taken from each bird with gross lesions. Samples were kept refrigerated or in buffered formalin solution for both bacteriological and histopathological diagnosis. Macroscopic characteristics and weight of livers were recorded for each bird.

Histopathology
Intestinal segments showing gross lesions compatible with NE were analyzed microscopically. Samples from duodenum, jejunum and ileum were fixed in 10% phosphate-buffered formalin, paraffin-embedded, sectioned, stained, and examined for microscopic lesions.

Figure 1: Jejunum gross changes of NE developed after the experimental infection. (A) Hemorrhages disseminated in the mucosae. The upper section shows a single hemorrhagic focus, 15 mm in size. The other segment has several coalescent foci (Score 3). Bar: 10 mm. (B) Numerous necrotic foci, many of those coalescent, accompanied by pseudomembranes were found in few animals (Score 5). Bar: 15 mm.
Results

High protein diet vs. regular diet

Initial attempts to reproduce NE lesions in broiler chickens without feed withheld produced lesions in less than 40% of the challenged birds consuming regular or high protein diets (data not shown). In subsequent experiments, birds were fasted for 12 hours before first dose of *C. perfringens* inoculum. In the high protein diet group, gross lesions (Figure 1) were observed in 62% of the challenged birds which was statistically different to the corresponding control group (12%; P<0.05). In the regular diet group, 63% of the challenged birds developed gross lesions which was statistically different to the corresponding control group (0%; P<0.05).

Differences in the average gross lesion score between challenged and unchallenged groups were only significant in the birds fed with regular diet. In birds fed with HPD, intestinal gross lesions were more severe in the challenged group (1.80 vs. 0.90; P>0.05). In birds fed with a regular diet, gross lesions were as well more severe in the challenged group (2.70 vs. 0.00; P<0.01). Based on our data, we were unable to demonstrate statistically that chickens fed with a HPD in addition to challenge with *C. perfringens* have increased odds of pathology compared with challenged birds under a regular protein diet. The association between “pathology” (present/absent) and exposure was assessed using a bivariate GLM. Results from experiments with chickens under HPD or regular diets challenged with *C. perfringens* are summarized in Table 1.

Necropsy and histopathology findings

Challenged birds necropsied three days after the initial challenge showed lesions in the jejunum and ileum (eventually in cecum) being more frequent in this last one (72% vs. 43%, considering all the independent assays performed with 60 birds) and with higher odds of injury in jejunum (OR=9.05, 95% CI=3.3-29.3, P < 0.0001) than ileum (OR=3.7, 95% CI=1.5-10.3, P=0.00741). In total, lesions were observed in 70% of the inoculated birds whereas none of the chicks in the control group developed gross lesions compatible with NE. Lesion score distribution for each portion of the small intestine are summarized in Tables 2 and 3.

Microscopic observation confirms the identity of recorded gross lesions. Microscopic lesions observed in tissue sections from the inoculated group included foci of necrosis, haemorrhage and epithelium desquamation. In more severe cases, accumulation of fibrinous exudate was observed (Figure 2). Mucosal smears of the small intestine of the inoculated group showed abundant short Gram positive bacilli compatible with *C. perfringens*. Although intestinal gross lesions were observed in the control group, microscopic changes were not compatible with NE lesions.

Abundant and pure growth of anaerobic bacilli compatible with *C. perfringens* was obtained from intestinal gross lesions samples of the inoculated group. The identity of the suspected colonies, randomly sampled, was confirmed by molecular and biochemical methods. *C. perfringens* colonies were not obtained from samples of the control group.

Table 1: Gross intestinal lesion frequency and scores of chickens experimentally challenged with *C. perfringens*.

<table>
<thead>
<tr>
<th>Diet (treatment)</th>
<th><em>C. perfringens</em> challenge</th>
<th>Odds Ratio</th>
<th>Birds with gross lesions/ total birds (%)</th>
<th>Average lesion score</th>
<th>Birds with lesions in jejunum (%)</th>
<th>Birds with lesions in ileum (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High protein diet</td>
<td>Inoculated</td>
<td>3</td>
<td>10/16 (62%)(^1)</td>
<td>1.8±0.82(^1)</td>
<td>10/10 (100%)</td>
<td>5/10 (50%)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td></td>
<td>2/16 (12%)(^1)</td>
<td>0.9±0.67(^1)</td>
<td>1/2 (50%)</td>
<td>1/2 (50%)</td>
</tr>
<tr>
<td>Regular diet</td>
<td>Inoculated</td>
<td>5.3</td>
<td>17/27 (63%)(^2)</td>
<td>2.7±0.5(^2)</td>
<td>17/17 (100%)</td>
<td>13/17 (76%)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td></td>
<td>0/25 (0%)(^2)</td>
<td>0(^2)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

\(^1,2\) Values with matching superscripts have statistically significant differences in percent of birds developing lesions (p<0.05).
Clinical signs consistent with NE in any of its presentations were not observed during the challenge although diarrhoea and also blood-streaked stool was commonly found in beddings and some of the birds in the inoculated groups showed signs of depression and reluctance to move after the challenge. In the control groups, no bird showed such changes at any time. No morphological alterations were observed in the liver of the inoculated birds; neither significant association was observed in any of the four linear regression models tested, each including liver weight (continuous) as dependent variable and: 1) global lesion score, 2) total lesion score, 3) jejunum score, and 4) ileum score, as independent variables in each of these models.

Weight gain and feed conversion

The effects of NE over bodyweight gain and food consumption was registered throughout a modified version of the trial. Because three days post challenge were not enough to observe change sat the production level, the trail was extended up to 15 days post challenge. At the end of this test, the control group achieved a greater average body weight gain compared to C. perfringens inoculated group (841.8 grs vs. 771.9 grs, see Figure 3). In the challenged group, an inverse correlation between weight gain and degree of injury was observed, particularly in birds with lesions of score 3 or higher, although it was not statistically significant. No differences were observed in total feed consumption between inoculated and control groups, although, feed efficiency was lower within the first group (1.76 vs. 1.71).

Discussion

NE is a complex, multifactorial disease with many unknown factors influencing its occurrence and the severity of outbreaks. Nearly all developed models of NE depend on the presence of predisposing factors, two of the most important being mucosal damage caused by coccidian pathogens [11,12] and feed containing high protein levels [6]. The experiments performed in the present report show a successful and consistent experimental reproduction of NE lesions in birds challenged during three days with C. perfringens cultures, with no significant differences either if high protein levels were or not added in the feed. These results show that NE can be reproduced by the sole administration of pathogenic C. perfringens with no other predisposing factor than feed withdrawal.

It has been described that C. perfringens strains derived from clinically healthy broilers or other animal species did not produce NE in broilers, even administering high numbers of C. perfringens cells in the gut and despite the use of predisposing factors [13]. Recent experiments show that only certain C. perfringens strains are capable to induce NE in chickens and that those strains normally constitute only a minority in the intestinal tract of healthy chickens. Cooper and Songer suggested that the potential of the strains isolated from field cases of NE to reproduce lesions diminishes with in vitro subculturing [6]. Also, we have observed a lower pathogenicity of NE strains after repeated in vitro subculturing (data not shown). Concordantly, previous works report that these C. perfringens NE isolates emerge as very specialized strains [13,14], which require determined virulence factors codified in plasmids or other mobile genetic elements to be fully pathogenic [15]. Therefore, the use of fresh or well preserved isolates of C. perfringens from cases of NE seems to be determinant to induce lesions during the experimental reproduction of NE in broilers. Also, an optimized culture protocol seems to be necessary for an effective C. perfringens challenge as Cooper and Songer stated that simplified

Table 2: Score distribution of jejunum gross lesions.

<table>
<thead>
<tr>
<th>Gross lesion score</th>
<th>Birds fed with regular diet(^1)</th>
<th>Birds fed with high protein diet(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inoculated</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>Inoculated</td>
<td>Control</td>
</tr>
<tr>
<td>0(^1)</td>
<td>0/17</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>3/17</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>4/17</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>5/17</td>
<td>0</td>
</tr>
<tr>
<td>n</td>
<td>17/17</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^1\)Birds with jejunum NE compatible gross lesions/ Birds with intestinal NE compatible gross lesions.

\(^2\)Gross lesion score =0 includes birds without evident NE compatible gross lesions.

Table 3: Score distribution of ileum gross lesions.

<table>
<thead>
<tr>
<th>Gross lesion score</th>
<th>Birds fed with regular diet(^1)</th>
<th>Birds fed with high protein diet(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inoculated</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>Inoculated</td>
<td>Control</td>
</tr>
<tr>
<td>0(^1)</td>
<td>0/17</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0/17</td>
<td>1/10</td>
</tr>
<tr>
<td>2</td>
<td>0/17</td>
<td>2/10</td>
</tr>
<tr>
<td>3</td>
<td>4/17</td>
<td>2/10</td>
</tr>
<tr>
<td>4-5</td>
<td>3/17</td>
<td>0</td>
</tr>
<tr>
<td>6(^2)</td>
<td>13/17</td>
<td>5/10</td>
</tr>
</tbody>
</table>

\(^1\)Birds with ileum NE compatible gross lesions/ Birds with intestinal NE compatible gross lesions.

\(^2\)Gross lesion score =0 includes birds without evident NE compatible gross lesions.
The 

**Conclusions**

Experimental conditions used in the present study, without the inclusion of severe changes on feed formulation (high protein levels) or other frequent predisposing factors as coccidia or presence of 

NetB-negative 

strains also produce NE [6]. On the other hand, Beta2 toxin may have a role in the pathogenesis of NE, but surveillance of healthy chickens and chickens with NE have not revealed a direct correlation between occurrence of disease and presence of cpb2 in isolates [20]. Therefore, further studies with isolates of diverse genetic background, including NetB toxin positive and negative strains are planned to compare virulence observed in the field and the NE model presented here.

References


Acknowledgements

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