RESEARCH NOTE

Research Note: *Clostridium perfringens* NetB and CnaA neutralizing nanobodies in feed reduce the severity of poultry necrotic enteritis

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ABSTRACT

Necrotic enteritis is a devastating disease to poultry caused by the bacterium *Clostridium perfringens*. As a novel approach to combating poultry necrotic enteritis, we identified and characterized several hundred single domain antibody fragments (or nanobodies) capable of binding either the NetB toxin or the collagen-binding adhesin (CnaA) of *C. perfringens*. Many of the nanobodies could neutralize the *in vitro* functions of NetB or CnaA with inhibitory concentrations in the nanomolar range. The nanobodies were also screened for proteolytic stability in an extract derived from gastrointestinal tract fluids of chickens. A collection of six nanobodies (four targeting NetB and two targeting CnaA) with high neutralizing activity and high gastrointestinal tract extract stability were expressed and secreted by *Pichia pastoris* or *Bacillus subtilis*. Chickens were given a feed with one of the two nanobody-containing groups: (1) nanobody-containing *P. pastoris* supernatants that were semi-purified, lyophilized, and enterically coated, or (2) *B. subtilis* spores from strains containing the nanobody genes. Chickens receiving either feed type had reduced necrotic enteritis disease severity in an experimental infection model. These results offer a new opportunity to improve the control of poultry necrotic enteritis by incorporating highly specific nanobodies or bacteria expressing these nanobodies directly into chicken feed.

Key words: *Clostridium perfringens*, necrotic enteritis, nanobodies, feed

INTRODUCTION

Poultry necrotic enteritis (NE) is a devastating gastrointestinal disease in commercial poultry production. The disease is estimated to cause a yearly global loss to producers of $6 billion USD and takes both a clinical form characterized by depression, diarrhea, anorexia, lesions in the small intestine and increased mortality, and a subclinical form characterized by damage to the mucosa of the small intestine and poor performance, but without increased mortality (Emami and Dalloul, 2021). The disease often requires a predisposing factor, such as *Eimeria* infection or dietary changes, that disrupts the physical properties of the gastrointestinal tract and allows for the proliferation of *Clostridium perfringens* strains that possess the NetB toxin (Moore, 2016).

Many products to reduce NE are available or in development (Abd El-Hack et al., 2022). These include vaccines, products to improve overall gut health (such as prebiotics, probiotics, essential oils, and phytochemicals), products that improve feed quality, products to control coccidiosis, and products to reduce immunosuppressive diseases. However, despite these options, NE continues to cause tremendous losses to poultry producers and requires the development of new interventions. We sought to apply an emerging technology to address this problem. Unlike most of the available products, we wanted a technology that could directly target the virulence factors of *C. perfringens* responsible for NE. Camelids, such as llamas, produce an unusual type of antibody consisting of only two heavy chains and the antigen binding V_HH domain of these antibodies can be recombinantly expressed as single-domain antibodies or nanobodies (Muyldermans, 2013). These nanobodies are the smallest antibody fragment that retains the entire antigen binding site of the original antibody from which they are derived, and they are being developed for a wide range of applications. We focused on two *C. perfringens*

virulence factors that play key roles in necrotic enteritis: the pore-forming toxin NetB and the collagen binding adhesin CnaA (Keyburn et al., 2008; Wade et al., 2016; Lacey et al., 2018) for the development of neutralizing nanobodies.

MATERIALS AND METHODS

Antigen and Nanobody Expression and Purification

NetB (GenBank Accession #ABW71134.1, amino acid residues S31-L322) was expressed as an N-terminal fusion with His_6-thioredoxin (TRX), cleavable with Tobacco Etch Virus protease (TEVp) in *Escherichia coli* Rosetta-gami 2 grown overnight in autoinducing media, 100 μg/mL ampicillin at 30°C. The CnaA collagen binding domain (GenBank Accession #ALJ54440.1, amino acid residues G35-K336) was expressed as an N-terminal fusion with His_6-maltose binding protein (MBP), cleavable with TEVp in *E. coli* BL21 grown overnight in LB broth, 1 mM IPTG, 25 μg/mL kanamycin at 30°C. After cell lysis via sonication, the soluble fraction was passed over a HisTrap HP column (Cytiva) and the bound protein eluted with an imidazole gradient (0 – 300 mM). After TEVp cleavage, the proteins were passed over a second HisTrap HP column and were collected in the flowthrough. The proteins were further purified with ion exchange and size exclusion chromatography using HiTrapQ HP (Cytiva) and Superdex 200 Increase 10/300 GL (Cytiva) columns, respectively, concentrated to ~10 mg/mL in a final buffer of 20 mM HEPES, 150 mM NaCl, pH 7.4, aliquoted, flash frozen, and stored at -80°C. Uncleaved CnaA was also produced for use in the functional assay described below.

Nanobodies were expressed and purified using the same process described for NetB above up to the second HisTrap HP column. They were further purified using cation (HiTrap SP HP, Cytiva) or anion (HiTrapQ SP, Cytiva) exchange chromatography depending on the pI,

concentrated to ~10 mg/mL in a final buffer of 20 mM HEPES, 150 mM NaCl, pH 7.4, aliquoted, flash frozen, and stored at -80°C.

Llama Immunization and Nanobody Discovery

Typical llama immunization and phage-based nanobody discovery protocols were used throughout these studies (Baral et al., 2013). Briefly, llamas were injected intramuscularly with 100 μg of each antigen on days 1, 21, 42, and 63. On days 28, 49, and 70, lymphocytes were collected, and RNA was isolated from the lymphocytes. RNA was converted to cDNA, and nanobody genes were PCR-amplified and used to create M13 phage libraries, with each phage particle in the library carrying a single nanobody DNA sequence internally and expressing the nanobody fused to the phage gIII tip protein. Phage libraries were enriched for antigen-specific binders through three rounds of biopanning. Finally, binding of individual monoclonal phages was quantified using an anti-M13 antibody in an ELISA assay. Nanobody genes from phage with four-fold greater binding on antigen-coated wells compared to PBS-coated wells were sequenced and those with unique complementarity determining regions were selected for *in vitro* characterization.

NetB Cytotoxicity Assay

Hepatocellular carcinoma-derived epithelial cells from *Gallus gallus* strain Leghorn (LMH cells) were adhered to the surface of tissue-culture treated and gelatin-coated 96-well microtitre plates at 64,000 cells/well in Waymouth's media + 10% FBS overnight at 37°C and 5% CO_2. 50 nM NetB was pre-incubated with 10-40,000 nM nanobodies or buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) for 15 minutes at 37°C and 5% CO_2. 11 μL of NetB/nanobody mixtures was applied to LMH cells in 100 μL of media and incubated for 5 hours at 37°C and 5% CO_2. Cytotoxicity induced by NetB was measured using the Pierce LDH Cytotoxicity Assay

Kit (Thermo Fisher Scientific) following the manufacturer's instructions. NetB cytotoxicity in the presence of a nanobody was determined relative to NetB cytotoxicity in the presence of buffer.

CnaA Collagen Binding Assay

Collagen type IV (Sigma -Aldrich, C7521) was dissolved in PBS at 20 μg/mL and coated on to wells of 96-well microtitre plates overnight at 4°C. The plates were washed with 200 μl of PBS and then blocked with 200 μl of 5% skim milk in PBS for 2 hours at 37°C. MBP-CnaA (100 nM) was pre-incubated with nanobodies (12.5 to 1600 nM) or buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) for 30 minutes at 37°C. The plates were washed 3x with 200 μl of PBS, and 100 μl of MBP-CnaA/nanobody mixtures were added to wells and incubated for 2 hours at 37°C. The plates were washed 3x with 200 μl of PBS and incubated with 100 μl of 0.125 μg/ml of anti-His antibody-HRP conjugate for 1 hour at room temperature. The plates were washed 3x with 200 μl of PBS and 100 μl of TMB substrate was added and developed for 30 minutes. 50 μl of 1 M HCl was added to each well to stop the reaction and A_{450} was measured. CnaA binding to collagen in the presence of a nanobody was determined relative to CnaA binding to collagen in the presence of buffer.

Gastrointestinal Tract Extract Stability Assay

Reactions were set up in volumes of 8 μL on ice. Each tube consists of 2.4 μL chicken jejunal extract, 5 μg nanobody in 0.8 μL PBS, and 4.8 μL of 150 mM NaCl. Control reactions without jejunal extract were also set up containing 5 μg nanobody in 3.2 μL PBS and 4.8 μL of 150 mM NaCl. The tubes were incubated on ice for 5 minutes, followed by 42°C for up to 24 hours. After incubation, 8 μL of preheated 2X SDS sample buffer was added to stop the reaction and nanobody stability was assessed using SDS-PAGE.

Production of P. pastoris Supernatants Containing Nanobodies

Individual nanobody genes were codon optimized for *Pichia pastoris* and nanobody proteins were secreted by *P. pastoris* strain BG11 with C-terminal 6X-histidine tags under the control of the methanol-inducible AOX promoter. Transformed cells were plated on yeast extract peptone dextrose (YPD) agar with 500 μg/ml zeocin and incubated for 2 days at 30°C. Twenty colonies for each nanobody were screened for nanobody secretion. The *P. pastoris* clone with the highest expression was chosen for fed batch fermentation. After fermentation, yeast biomass was removed using centrifugation and nanobodies were enriched from supernatants using three rounds of ultrafiltration with a 2 kDa molecular weight cut off. Filtered supernatants for each supernatant were frozen and lyophilized.

Production of B. subtilis Spores Expressing Nanobodies

Individual nanobody genes were codon optimized for *Bacillus subtilis* and nanobody proteins were secreted by *B. subtilis* strain PY79 with C-terminal 6X-histidine tags under the control of the constitutive P_{veg} promoter (Lam et al., 1998). Based on small-scale expression tests, the clone with highest expression was selected for spore production. Cells were grown in 1 L of Difco sporulation media for 72 hours and harvested by centrifugation. Pellets were air dried and ground into a powder. Spores/g were quantified by colony counting after heating samples (80ºC for 20 minutes) to inactivate vegetative cells.

Experimental Necrotic Enteritis Infection of Chickens

Five hundred and sixty chicks (day-of-hatch; day 0) were assigned to seven treatment groups with eight cages per treatment and ten chicks per cage. Treatment groups were assigned to cages using a randomized complete block design. The study began when chicks were placed in experimental cages. Only healthy birds were selected. Chicks were raised in Petersime style

147 battery cages (stocking density of 0.63 square feet per bird) in a solid-sided barn and maintained
148 under ambient humidity. Feed and water were available *ad libitum* throughout the trial. Each
149 cage contained one trough feeder and one trough drinker (10 bird to feeder/ drinker ratio, 24-inch
150 x 3.5-inch trough). All birds were weighed by cage on days 0, 14, 22, and 28. The necrotic
151 enteritis challenge model consisted of approximately 2,500 *Eimeria maxima* oocysts gavaged on
152 day 14 as the predisposing factor and *C. perfringens* strain CP4 gavaged on days 19 and 20 in 1.0
153 mL at concentrations of 3.0×10^8 and 7.0×10^8 CFU/mL, respectively. *B. subtilis* spores were
154 fed to chicks throughout the entire experiment at a dose of 6×10^9 CFU/kg feed. Enterically
155 coated yeast supernatants containing nanobodies were fed to chicks from day 18 to day 28 of the
156 experiment at a dose of 5.3 g/kg feed. One control group received bacitracin methylene
157 disalicylate (BMD) at a dose of 55 mg/kg feed an industry standard antibiotic treatment, from
158 day 18 to day 28. Necrotic enteritis mortality in birds that died during the trial was assessed by
159 gross lesions during necropsy. The trial was terminated on day 28. Statistical analysis was
160 conducted with the STATISTIX for Windows software. Groups were compared by ANOVA with
161 a comparison of means using least significant difference (LSD) test and were considered
162 statistically different at $P < 0.05$. The *in vivo* efficacy study was conducted at Southern Poultry
163 Research Group (Athens, GA). Animal care practices adhered to the Guide for the Care and Use
164 of Agricultural Animals in Agricultural Research and Teaching (Federation of Animal Science
165 Societies).

166 **RESULTS AND DISCUSSION**

167 ***Selection of Lead Nanobodies***

168 A total of six llamas were inoculated with NetB and CnaA, resulting in the discovery of
169 192 and 217 unique nanobodies to the two antigens, respectively. More than 90% of these

nanobodies were successfully purified from *E. coli* expression and screened in antigen-specific *in vitro* functional assays and stability assays.

All α-NetB nanobodies were screened for their ability to reduce the cytoxicity of NetB to tissue cultured LMH cells. Fig. 1A shows the results for four nanobodies (NBX-NB1, NBX-NB2, NBX-NB3, and NBX-NB4) that could reduce NetB cytotoxicity by >90% and had average IC_{50} values below 100 nM. The mean (and standard error of the mean) from four independent experiments for these nanobodies were: NBX-NB1, 33 ± 5.7 nM; NBX-NB2, 71 ± 10 nM; NBX-NB3, 8.4 ± 2.3 nM; NBX-NB4, 4.6 ± 0.3 nM.

All α-CnaA nanobodies were screened for their ability to reduce the binding of CnaA to collagen. Fig. 1B shows the results for two nanobodies (NBX-CA1 and NBX-CA2) that could reduce binding of CnaA by >50% and had average IC_{50} values below 200 nM. The mean (and standard error of the mean) from four independent experiments for the two nanobodies were: NBX-CA1, 184 ± 20 nM; NBX-CA2, 169 ± 25 nM.

All nanobodies were screened for proteolytic stability in a chicken jejunal extract. Fig. 1C shows the results for the six nanobodies described above. All six were visible on SDS-PAGE for at least one hour in the presence of the extract. Of the more than 400 α-NetB and α-CnaA nanobodies that were discovered and screened in this assay; the six nanobodies shown here are in the top 12.5% of the most stable proteolytically stable nanobodies in chicken gastrointestinal tract extracts.

Many other nanobodies had either high efficacy in the *in vitro* functional assays or high proteolytic stability. The six nanobodies described above had the best combination of *in vitro* function and stability, and thus were selected for use in *in vivo* studies as a collection.

Production of Materials for In Vivo Trial

193 The quality and quantity of nanobodies in the lyophilized yeast supernatant were
194 determined before *in vivo* studies. To do this, a small amount of each powder was resuspended in
195 buffer and the soluble fraction was used for characterization. Nanobody activity were maintained
196 in the *in vitro* function assays described above for all six lead nanobodies (data not shown).
197 Nanobody concentrations in the powders were estimated by comparing dissolved material to a
198 standard curve of purified protein using SDS-PAGE analysis. After mixing materials for the six
199 lead nanobodies, the total nanobody content in the lyophilized yeast supernatant was estimated to
200 be~11% by weight. The mixed lyophilized yeast supernatants were enterically coated using a
201 lipid-based matrix (Jefo Nutrition) with a final inclusion rate of 18%. A similar, enterically
202 coated mixture of off target nanobodies that are not expected to reduce necrotic enteritis was also
203 produced with a final inclusion rate of 12%.

204 ***In Vivo Efficacy Trial***

205 Results of the *in vivo* efficacy trial are shown in Table 1. Unchallenged chicks (Group 1)
206 showed no necrotic enteritis mortality, while challenged/untreated chicks (Group 2) had 23.75%
207 mortality. Both treatments containing active nanobodies (Groups 4 and 6) reduced mortality to
208 levels equal to or lower than the BMD (antibiotic) control (Group 3). There were also decreases
209 in mortality in the groups receiving off-target nanobodies (Group 5) or *B. subtilis* without
210 nanobodies (Group 7); however, the reductions were not as great as those groups that received
211 active nanobodies targeting NetB and CnaA, indicating that the nanobodies play an active role in
212 disease reduction. It is possible that the materials in the encapsulant and the *B. subtilis* bacteria
213 themselves also contribute some overall benefit to gut health and account for a portion of the
214 improvement seen with the nanobody-containing groups.

Performance parameters were also measured throughout the 28-day challenge. Once adjusted for mortality, the best feed conversion ratio improvements compared to challenged/untreated chickens (Group 2) were for the two groups that contained active nanobodies (Groups 4 and 6). Both groups outperform the feed conversion ratios seen in the BMD control (Group 3). Like the observation above for necrotic enteritis mortality, the control treatments lacking active nanobodies (Groups 5 and 7) also have some benefits to performance parameters compared to challenged/untreated chickens and may contribute to some of the benefit seen in the nanobody-containing groups. However, the data indicate that the active nanobodies make the most substantial improvements to feed conversion ratios.

To the best of our knowledge, this is the first study to indicate that nanobodies can reduce poultry necrotic enteritis when they are applied in poultry feed and when they are applied in a recombinantly expressed and semi-purified form. In contrast, a previous demonstration of the efficacy of nanobodies for poultry necrotic enteritis was restricted to a bacterial delivery system through *in ovo* and water administration (Gangaiah et al., 2022). Although different application methods may be useful under certain conditions, the ability to incorporate the material directly into poultry feed provides a very straightforward route of administration to farmers.

Despite several commercially available products for necrotic enteritis reduction, this disease remains a significant challenge to poultry producers and continues to lead to billions of dollars in losses around the world every year. The data presented here offer a new strategy for a highly specific and targeted approach that can be directly incorporated into poultry feed as a prophylactic against necrotic enteritis. The possibility also exists to utilize these molecules therapeutically as an alternative to or in conjunction with an antibiotic.

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281 Table 1. *In vivo* efficacy trial results

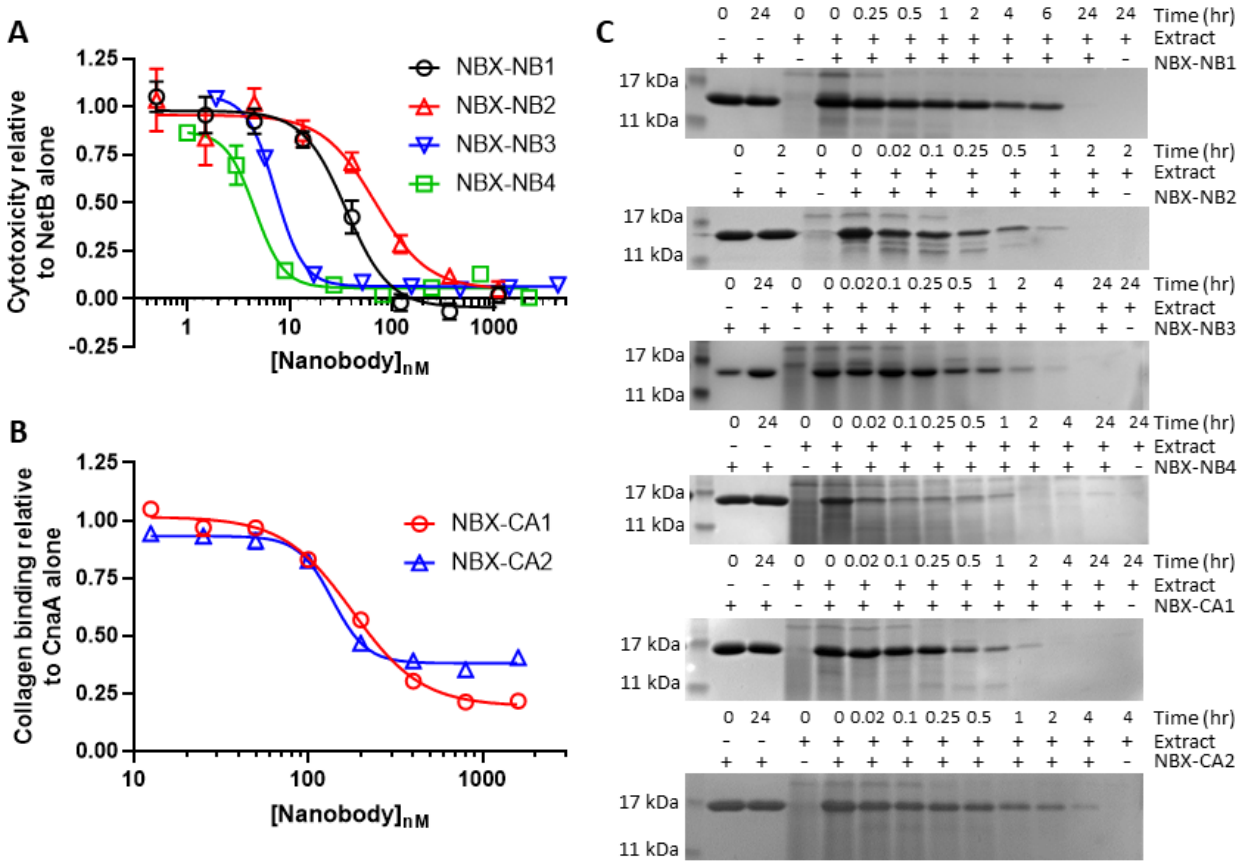
Group #	Description	Necrotic Enteritis Percent Mortality*	Non-Adjusted Feed Conversion Ratio*,+	Adjusted Feed Conversion Ratio*,+
1	Unchallenged	0.00^{c}	1.437^{c}	1.437^{c}
2	Challenged, Untreated	23.75^{a}	1.972^{a}	1.607^{a}
3	Challenged, BMD treated	$11.25^{a,b,c}$	$1.691^{a,b,c}$	$1.565^{a,b}$
4	Challenged, Active nanobody treated	$11.25^{a,b,c}$	$1.698^{a,b,c}$	$1.540^{a,b}$
5	Challenged, Off-target nanobody treated	$17.5^{a,b}$	$1.806^{a,b}$	$1.567^{a,b}$
6	Challenged, *B. subtilis* secreting active nanobody treated	$7.5^{b,c}$	$1.564^{b,c}$	$1.485^{b,c}$
7	Challenged, *B. subtilis* treated	$15^{a,b}$	$1.757^{a,b}$	1.585^{a}

282 *Means within a column lacking a common superscript differ ($P < 0.05$)

283 $^{+}$Day 0 to Day 28 Performance Results

Figure Legends

Fig. 1: *In vitro* characterization of lead nanobodies. (A) Four α-NetB nanobodies (NBX-NB1, NBX-NB2, NBX-NB3, and NBX-NB4) block the cytotoxicity of NetB to tissue cultured LMH cells in a dose-dependent fashion. Data shown is from a representative experiment for each nanobody. Data points are the mean and standard error of the mean from triplicate wells. (B) Two α-CnaA nanobodies (NBX-CA1 and NBX-CA2) reduce the binding of CnaA to collagen in a dose-dependent fashion. Data shown is from a representative experiment for each nanobody. Data points are the mean and standard error of the mean from triplicate wells. (C) All six nanobodies survive for an hour or more in the presence of a chicken jejunal extract. For each nanobody, the SDS-PAGE gel shows the following from left to right: 11 and 17 kDa molecular weight markers; the nanobody, incubated at 42ºC in the absence of extract at the start and endpoints of the experiment to show that any observed degradation is extract dependent; the extract without nanobody at the start of the experiment to aid in the identification of the nanobody on the gel; a time course of the nanobody incubated at 42ºC in the presence of the extract; and the extract without nanobody at the end of the experiment to aid in the identification of the nanobody on the gel.


A
Cytotoxicity relative to NetB alone
1.25
1.00
0.75
0.50
0.25
0.00
-0.25
1
10
100
1000
[Nanobody]nM
NBX-NB1
NBX-NB2
NBX-NB3
NBX-NB4
B
Collagen binding relative to CnaA alone
1.25
1.00
0.75
0.50
0.25
0.00
10
100
1000
[Nanobody]nM
NBX-CA1
NBX-CA2
C
Time (hr)
Extract
NBX-NB1
17 kDa
11 kDa
NBX-NB2
NBX-NB3
NBX-NB4
NBX-CA1
NBX-CA2

301