Supplemental Material

Supplemental Methods:

Construction of expression plasmids

Fragments consisting of GtxA, GtxA-C, GtxA-N, RTX, and RTX-C were PCR-amplified from the *G. anatis* genome, which was extracted as previously described (1) (primers listed in Table S2). The PCR fragments were purified using a MiniBest Agarose Gel DNA Extraction Kit (TaKaRa Bio Inc., Kusatsu, Shiga, Japan), according to the manufacturer's instructions, double digested with NcoI and XhoI, and gel purified again. Plasmids encoding these five GtxA mutants were constructed by ligating the different PCR fragments into the expression vector pET28a (Novagen, EMD Millipore Corp., Billerica, MA, USA). Plasmid pET28a was double-digested with NcoI and XhoI and gel-purified. The vector and the PCR fragments were ligated at a ratio of 1:3, transformed into DH5α chemically competent *E. coli* (Enzynomics, Daejeon, Republic of Korea), and selected on LB agar plates with kanamycin. The sequence of the insert in each plasmid was verified by nucleotide sequencing using an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). Plasmids containing the insert were also verified by double digestion and PCR amplification with the primers listed in Table S2. The plasmids were transformed into T7 express competent *E. coli* (New England Biolabs, Ipswich, MA, USA).

Expression and purification of recombinant GtxA-N protein

The T7 express competent *E. coli* containing the transformed plasmid with the GtxA-N insert was plated on LB agar containing 0.1 mM IPTG and incubated at 30°C overnight. A single colony was diluted in the LB broth at a ratio of 1:50 and incubated at 37°C with 200 rpm/min shaking until the culture reached an OD_{600} of 0.6. Then 0.2 mM IPTG was added and the culture was maintained at 30°C for 2 h. *The E. coli* was centrifuged and resuspended in ice-cold 1 × T7·Tag bind/wash buffer from a T7·Tag Affinity Purification Kit (Novagen). The recombinant GtxA-N protein was extracted and purified according to the manufacturer's instructions. After dialysis, the purified protein was further concentrated by sprinkling solid polyethylene glycol on the dialysis tubing. The purified recombinant protein concentration was determined using a Bicinchoninic Acid Protein Assay Kit (Sigma-Aldrich) with bovine serum albumin as the standard.

REFERENCES

1. Andreou LV. 2013. Preparation of Genomic DNA from Bacteria, p 143-151. *In* Lorsch J (ed), Methods in Enzymology, vol 529. Academic Press.

Supplemental Data:

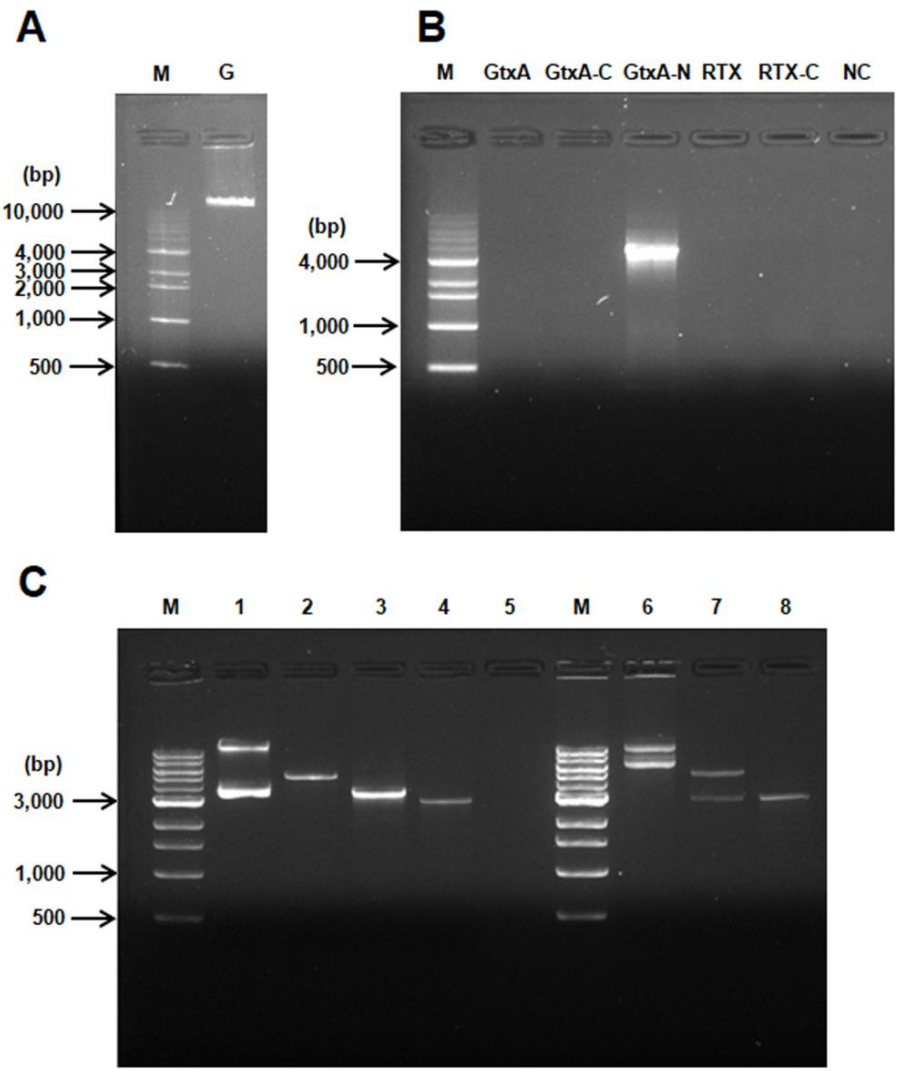

FIG S1 Construction of the GtxA-N expression plasmids. (A) *G. anatis* genomic DNA. M, 1 kb DNA marker; G, genomic DNA. (B) *G. anatis* GtxA gene PCR product. NC, negative control. (C) Analysis of recombinant plasmid by restriction enzymes and PCR amplification. Lane 1, pET28 vector; lane 2, pET28 vector digested with NcoI and XhoI; lane 3, the amplified product of GtxA-N from *G. anatis* genomic DNA; lane 4, GtxA-N PCR product digested with NcoI and XhoI; lane 5, PCR negative control; lane 6, pET28-GtxA-N; lane 7, pET28-GtxA-N digested with NcoI and XhoI; lane 8, the amplified product of GtxA-N from recombinant plasmid DNA.

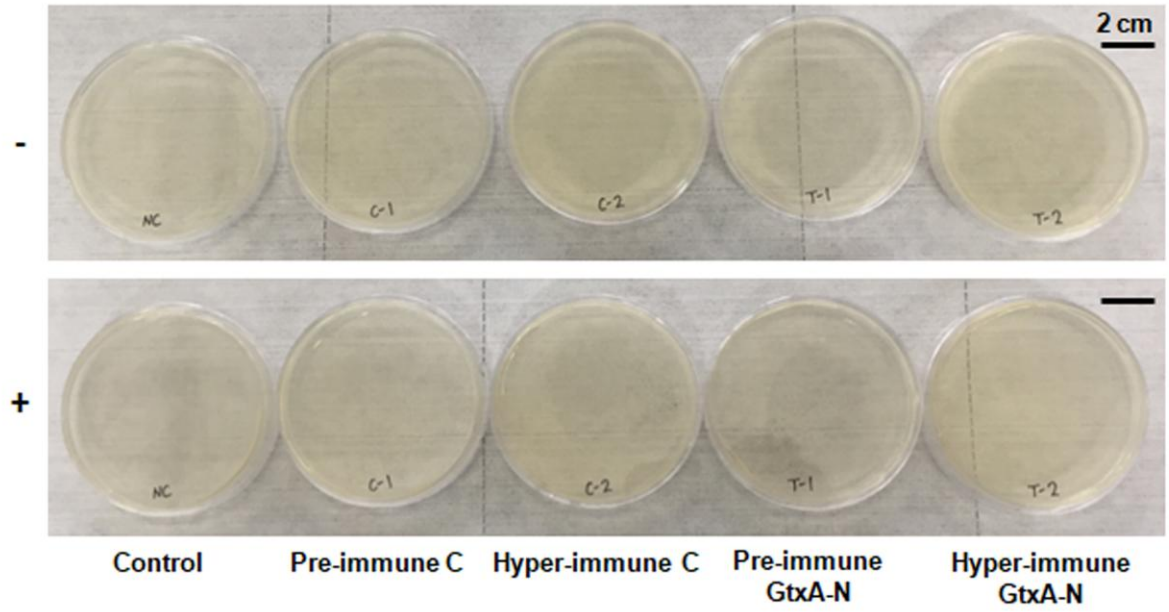

FIG S2 Sterility test results for the purified IgY antibody. –, no IgY; +, 48 h after IgY plating. Scale bar: 2 cm.

TABLE S1 Lesion scores in the peritoneum, liver, and duodenum and the total lesion scores

Organ	Individual	Chicks	Lesion scores			
			Pre-immune C	Hyper-immune C	Pre-immune GtxA-N	Hyper-immune GtxA-N
Peritoneum	1	#1	9	7	7	3
		#2	7	8	9	5
		#3	11	6	5	3
		#4	8	9	6	1
		#5	10	5	8	4
	2	#1	9	8	8	4
		#2	8	9	10	5
		#3	10	8	6	3
		#4	7	13	7	2
		#5	11	8	9	6
Liver	1	#1	3	2	2	1
		#2	1	1	1	1
		#3	5	3	3	2
		#4	2	4	2	0
		#5	4	0	4	3
	2	#1	4	5	5	2
		#2	2	3	3	1
		#3	6	7	4	5
		#4	3	6	3	1
		#5	5	2	6	3
Duodenum	1	#1	2	4	4	2
		#2	3	2	2	1
		#3	1	6	6	3
		#4	1	2	1	1
		#5	4	4	7	1
	2	#1	4	4	5	3
		#2	3	3	3	1
		#3	3	9	7	5
		#4	3	3	4	1
		#5	7	4	6	3
Total	1	#1	14	13	13	6
		#2	11	11	12	7
		#3	17	15	14	8
		#4	11	15	9	2
		#5	18	9	19	8
	2	#1	17	17	18	9
		#2	13	15	16	7
		#3	19	24	17	13
		#4	13	22	14	4
		#5	23	14	21	12

Two individuals performed the double-blind scoring of lesions in the peritoneum, liver, and duodenum for each chick, according to the description of each score within all parameters in Table 1.

TABLE S2 Primers used for construction, verification, and expression of GtxA mutants

Primer name	Sequence	Construct	Primers used
GtxAC1	AGTCCCATGGGTCTTTCATTAAAAGAAAAAGTAACTGGAATA	GtxA	GtxAC1 & GtxA-RTX2
GtxAC2	CAGTCTCGAGTTATGAATTTTCTTCTATAAAAGCAGC	GtxA-C	GtxAC1 & GtxAC2
GtxA-N	AGTCCCATGGCAATTGAATCTTTCAATTTAATCGCAA	GtxA-N	GtxAC1 & GtxA-RTX1
GtxA-RTX1	CAGTCTCGAGTTAATTTAGGAAATCGGTCATTATGCCAT	RTX	GtxA-N & GtxA-RTX2
GtxA-RTX2	CAGTCTCGAGTTAAACAAGATACATAGTGACCAGTTCAT	RTX-C	GtxA-N & GtxAC2

Restriction sites are underlined.