Design, Expression, and Testing of a Bivalent Subunit Botulism Vaccine for Use in Cattle

Thesis submitted by

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Abstract

Botulism intoxication in cattle is a serious problem that needs to be addressed. Current vaccines offer protection but are based on lysed cells and inactivated toxin. These formulations are dangerous to produce and vary widely between batches. A subunit vaccine based on non-toxic components of the botulinum neurotoxin would lead to safer vaccines, less *in vivo* testing and reduced costs.

The aims of the study were threefold. Genes encoding the non-toxic subunits of *Clostridium botulinum* serotypes C and D neurotoxins were to be redesigned and synthesized *de novo* to optimize expression in the methylotrophic yeast *Pichia pastoris*. The synthetic genes were to be transformed and expressed in the yeast and conditions determined for effective expression of both subunits. Finally the expressed proteins were to be tested *in vivo* in BALB/c mice to validate the completed protein expression work. Ultimately, the aim was to create a system that expressed high levels of both subunits C and D that were shown to be effective *in vivo*. This would then be transferred to industry and would lead to the commercialization of a new generation bivalent vaccine against botulism to be used on cattle.

The genetic work carried out yielded two genes that were codon optimized for expression in *P. pastoris*, had a higher GC content than the original sequence and whose protein products would not be glycosylated during secretion, owing to the targetted substitution of histidine codons for asparagines codons. The GC content was increased by 13% in the D subunit and by nearly 20% in the C subunit.

Yeast transformation and expression were carried out and led to the isolation of positive clones expressing the synthetic genes. Protein was isolated from both the cell pellet and the medium, indicating that the product was being secreted from the cells. Yields varied and reached nearly 14.5 mg/L for BoNT/C and more than 3 mg/L in the case of BoNT/D.

The *in vivo* trials tested the immunizing properties of the two expressed subunits. A number of conditions and parameters were tested in the mice and it was determined that the aluminium hydroxide ($Al(OH)_3$) adjuvant was the most effective adjuvant in terms of eliciting an immune response and leading to immunity in mice. Up to 60% of the mice immunized with subunit C were protected against a challenge of up to 25 LD_{50} but more than that proved fatal to naïve and immunized mice alike. ELISA and Western hybridizations indicated that antibodies were raised in the mice, especially when the aluminium hydroxide adjuvant was included in the formulation.

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Abbreviations

µg	microgram
µl	microlitre
APS	ammonium persulphate
BMG	buffered minimal media with glycerol
BMGY	buffered minimal media with glycerol and yeast extract
BMM	buffered minimal media with methanol
BMMY	buffered minimal media with methanol and yeast extract
BoNT	botulinum neurotoxin
bp	base pairs
BSA	bovine serum albumin
cDNA	complementary DNA
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EDTA	disodium ethylene diamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
g	gram
H_c	carboxy 50 kDa terminal of neurotoxin heavy chain
HCl	hydrochloric acid
His	histidine
HRP	horseradish peroxidase
kDa	kilodaltons
LB	Luria broth
LD_{50}	50% lethal dose
M	molar
Ni-NTA	nickel nitrilo-tri-acetic acid
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PVDF	polyvinylidenedifluoride
RNA	ribonucleic acid
RPM	revolutions per minute
SDS	sodium dodecyl sulphate
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptors
TBST	tris buffered saline with tween 20
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
YPD	yeast extract/ peptone/ dextrose

"Fill the gut of a sheep with blood taken from the carotid arteria of a black bull, tighten both ends, and hang it in the shadow of a mulberry tree. When dried grind the content firmly. A pinch of the powder mixed with food will kill the consumers within three days."

(A practical guide to the manufacturing process for botulinum neurotoxin.)

Schanaqua - Indian poison expert - Circa 1600

(Bigalke and Rummel, 2005)