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## Evaluating the potential of an *In vitro* expression system in generating difficult to-express Hc botulinum vaccines

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Botulinum neurotoxins produced by the anaerobic bacteria *Clostridium botulinum* are the most potent biological toxins in nature. Current vaccine strategies focus on the carboxy-terminal fragment of the neurotoxin (Hc) that contains most of the neutralizing epitopes. However, due to the high AT-content of clostridial genes, expression of recombinant Hc proteins in cellular systems is challenging, and was demonstrated to require codon-optimized synthetic gene. We used an alternative strategy in which the native genes encoding Hc proteins of botulinum toxins A, B and E were expressed in a cell-free expression system. The unique property of this open system was used to optimize solubility and expression level by introducing various chaperones, directly to the expression reaction. Expression levels of more than 1mg/ml were obtained in the continuous-exchange mode of the cell-free system. The purified Hc proteins were detected by anti-homologue toxin sera suggesting that native immunogenic epitopes are presented on the *in vitro*-expressed proteins. Mice immunized with, alum-absorbed, HcA, HcB or HcE vaccines generated a high serum ELISA-titers against the homologue native toxin complex, which enabled protection against a high-dose toxin challenge ( $10^3$ - $10^6$  MsLD<sub>50</sub>). Moreover, immunization with a trivalent HcA, HcB and HcE vaccine protected mice against the corresponding trivalent toxin challenge ( $10^5$  MsLD<sub>50</sub>). Finally, the anti Hc sera were used to develop an ELISA for specific detection of native botulinum A, B or E. Our results together with the latest developments in scalability of the *in vitro* expression systems offer alternative routes for the preparation of difficult to express sub-unit vaccines.