PRESENTATION TO GUTHY JACKSON CHARITABLE FOUNDATION

April 2019

Executive Summary

First Evidence of Epsilon Toxin in Neuromyelitis Optica Patients

There is an increasing body of published research indicating that a toxin, epsilon toxin, is associated with a significant minority of cases of multiple sclerosis (MS). Epsilon toxin is produced by two types (B and D) of a common bacteria, Clostridium perfringens.

The evidence shows positive correlations in MS patients of between 23% and 40% in a range of different tests for the toxin or antibodies to the toxin compared with 8% to 20% for matched controls.

The question we wanted to address was whether the same toxin could be evident in patients with neuromyelitis optica (NMO)?

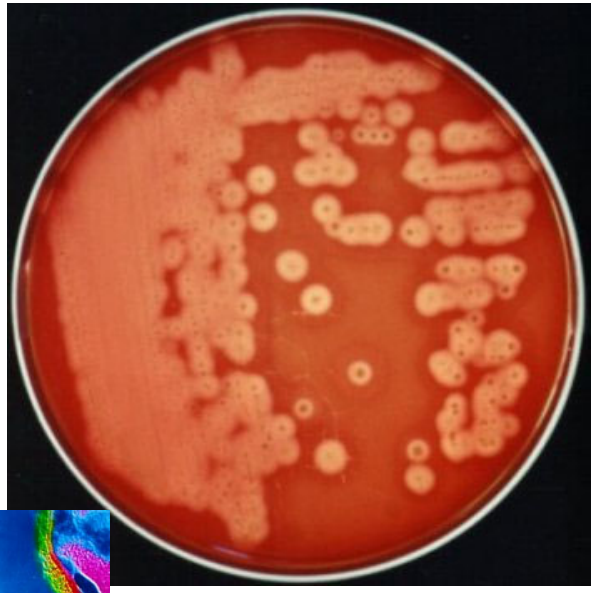
Preliminary results from a study of 30 NMO patients indicated a much higher incidence of antibodies to MS patients – 15 of 30 (50%) of the NMO samples tested positive to epsilon antibodies in their serum. Not only was the incidence higher for NMO patients, 3 of 30 (10%) showed exceptionally strong responses and even more so than any of the positives in the MS cohort.

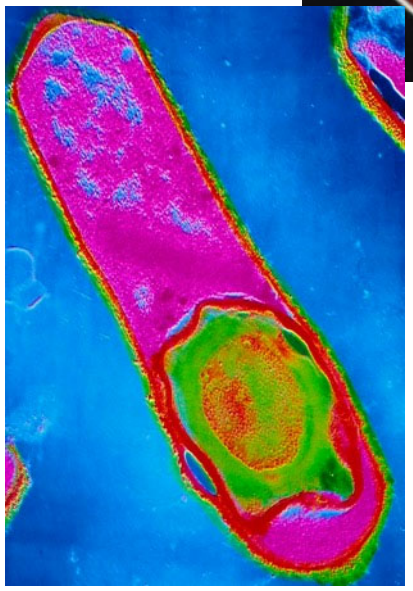
This evidence is exciting as it is leading towards identifying the possible root cause of both NMO and MS, which is a necessary start to finding a cure.

The next step is to repeat the experiment with a larger sample size on a longitudinal study with the assistance of the Guthy Jackson Charitable Foundation using its Circles Biobank.

What is epsilon toxin?

- Epsilon toxin (ETX) is produced by two types (B and D) of a common bacteria *Clostridium perfringens*
- 5 biotypes of *C. perfringens* (A,B,C,D and E)
- ETX is produced by types B & D and is a common causes of serious disease in livestock
- ETX has not previously been implicated in disease of humans



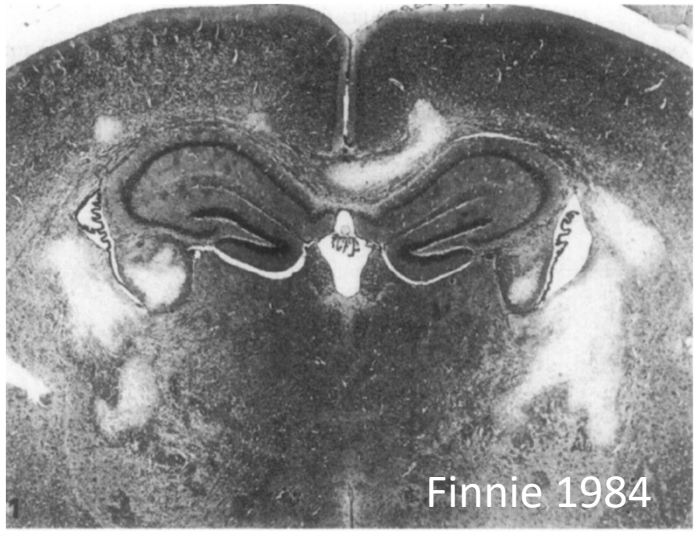


Epsilon toxin causes enterotoxaemia in livestock

- Affects mainly sheep, lambs and goats. Less often calves and other species
- Toxin produced in the intestine
- Crosses the gut wall.
- Causes damage in the kidneys and brain
- Rapidly fatal

Epsilon toxin is known to be neurotoxic in animals

- Rodents dosed with ETX show bilateral lesions
- ETX dosing causes aquaporin 4 up-regulation
- ETX damages oligodendrocytes
- ETX breaches blood-brain barrier



Vet Pathol 45:307–309 (2008)

BRIEF COMMUNICATIONS and CASE REPORTS

Aquaporin-4 in Acute Cerebral Edema Produced by *Clostridium perfringens* Type D Epsilon Toxin

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Abstract. Sheep, particularly lambs, with high circulating levels of *Clostridium perfringens* type D epsilon toxin develop severe neurologic signs and often die suddenly. On microscopic examination, in the brain, there is microvascular endothelial injury and diffuse vasogenic edema. The aquaporin (AQP) family of membrane water-channel proteins, especially AQP-4, is important in the regulation of water balance in the brain and facilitates reabsorption of excess fluid. In rats given epsilon toxin, generalized cerebral edema was demonstrated by marked albumin extravasation and was correlated with widespread upregulation of AQP-4 in astrocytes. These results suggest that AQP-4 has a role in the clearance of edema fluid from brains damaged by this clostridial toxin.

Key words: Aquaporin-4; cerebral edema; *Clostridium perfringens* type D epsilon toxin; rats.

Clostridium perfringens type D enterotoxemia is an important disease of sheep worldwide; the principal clinicopathologic manifestations are neurologic. Absorption into the circulation of large quantities of epsilon toxin produced by these proliferating intestinal bacteria after starch overload causes microvascular

tion, the brains were fixed by transcardiac perfusion with 4% paraformaldehyde that contained 0.02% heparin. After remaining in situ for 2 hours, the brains were removed and immersion-fixed in 10% buffered formalin for 4 days. Brains were then processed to paraffin wax, and 6-µm sections were cut and stained

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New insights into *Clostridium perfringens* epsilon toxin activation and action on the brain during enterotoxemia

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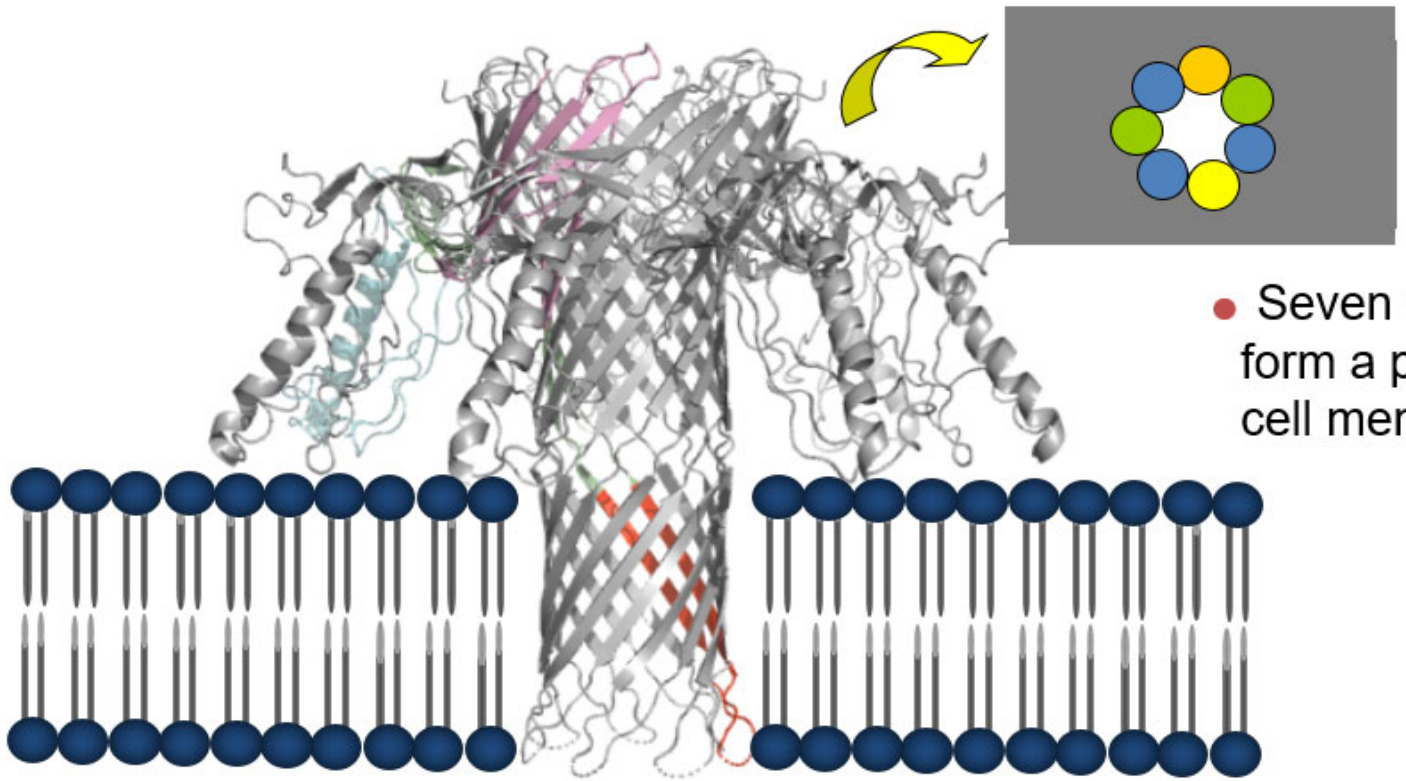
ABSTRACT

Epsilon toxin (ETX), produced by *Clostridium perfringens* types B and D, is responsible for diseases that occur mostly in ruminants. ETX is produced in the form of an inactive prototoxin that becomes proteolytically-activated by several proteases. A recent *ex vivo* study using caprine intestinal contents demonstrated that ETX prototoxin is processed in a step-wise fashion into a stable, active ~27 kDa band on SDS-PAGE. When characterized further by mass spectrometry, the stable ~27 kDa band was shown to contain three ETX species with varying C-terminal residues; each of these ETX species is cytotoxic. This study also demonstrated that, in addition to trypsin and chymotrypsin, proteases such as carboxypeptidases are involved in processing ETX prototoxin. Once absorbed, activated ETX species travel to several internal organs, including the brain, where this toxin acts on the vasculature to cross the blood-brain barrier, produces perivascular edema and affects several types of brain cells including neurons, astrocytes, and oligodendrocytes. In addition to perivascular edema, affected animals show edema within the vascular walls. This edema separates the astrocytic end-feet from affected blood vessels, causing hypoxia of nervous system tissue. Astrocytes of rats and sheep affected by ETX show overexpression of aquaporin-4, a membrane channel protein that is believed to help remove water from affected perivascular spaces in an attempt to resolve the perivascular edema. Amyloid precursor protein, an early astrocyte damage indicator, is also observed in the brains of affected sheep. These results show that ETX activation *in vivo* seems to be more complex than previously thought and this toxin acts on the brain, affecting vascular permeability, but also damaging neurons and other cells.

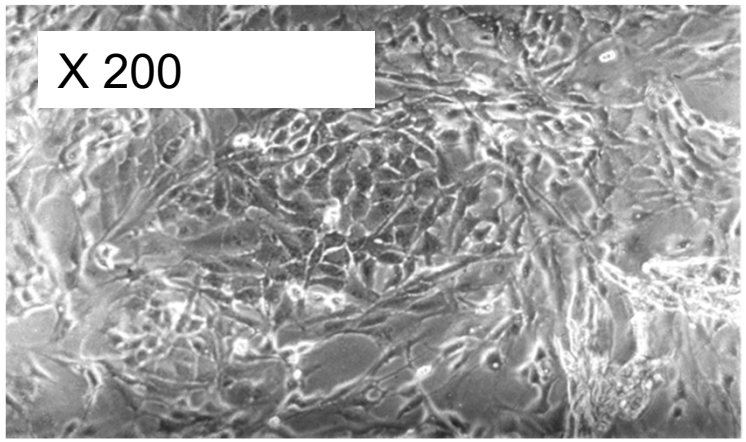
Epsilon toxin is toxic because it forms pores in target cells



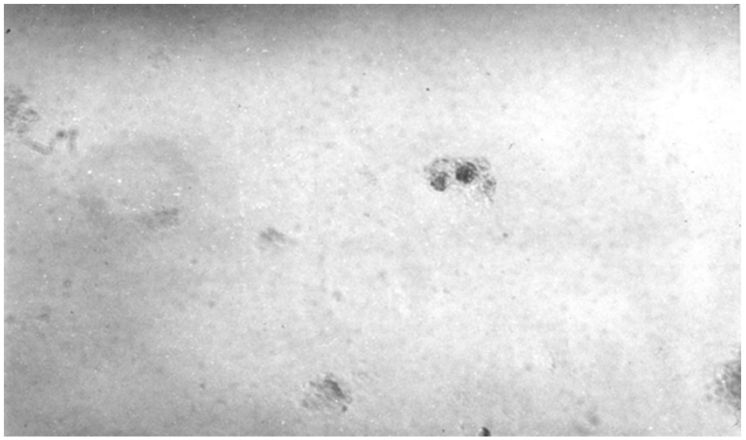
- Seven monomers form a pore in the cell membrane

Epsilon toxin is active only towards neuronal and kidney cells

- Most established cell lines (e.g CHO cells) are completely resistant to the toxin
- A few kidney cells lines are susceptible e.g. Madin Darby Canine Kidney (MDCK) cells



Control MDCK culture



MDCK cells + toxin

Myelin and lymphocyte (MAL) protein is the receptor for epsilon toxin

PLOS | PATHOGENS

RESEARCH ARTICLE

The Myelin and Lymphocyte Protein MAL Is Required for Binding and Activity of *Clostridium perfringens* ε-Toxin

Kareem Rashid Rumah[1,2☯], Yinghua Ma[1☯], Jennifer R. Linden[1☯], Myat Lin Oo[1], Josef Anrather[1], Nicole Schaeren-Wiemers[3], Miguel A. Alonso[4], Vincent A. Fischetti[2], Mark S. McClain[5], Timothy Vartanian[1]*

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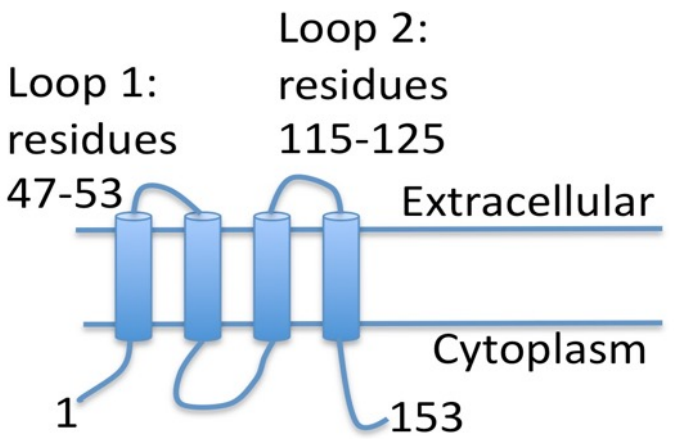
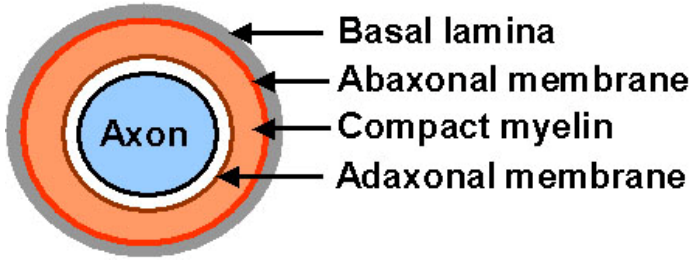


- MAL is localised in compact myelin

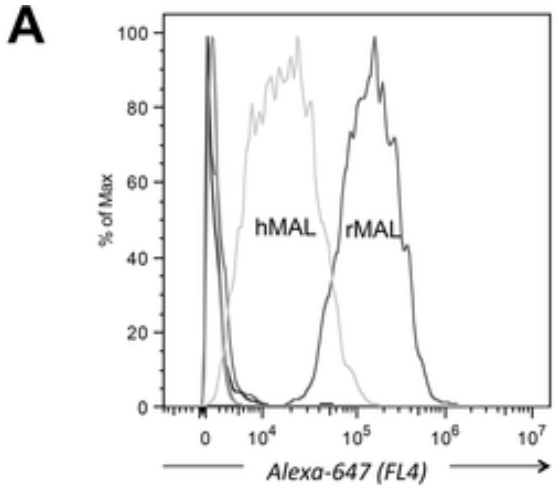


- MAL is also found in T-cells, some epithelial cells (e.g. in kidney, stomach, thyroid gland), oligodendrocytes, optic nerve and blood-brain barrier
- Highly hydrophobic tetra span membrane proteolipid with 2 extracellular loops

CHO cells become sensitive to epsilon toxin if they are engineered to express myelin and lymphocyte protein

Mutant	CT_{50} MDCK cells	CT_{50} CHO-cell expressing human MAL	CT_{50} CHO-cell expressing sheep MAL	CT_{50} CHO-cell expressing dog MAL
Wild type toxin	9.7nM	12.7nM	1.6nM	1.7nM
Y43AY209A	1.6 μM	5.5 nM	13.75nM	306nM

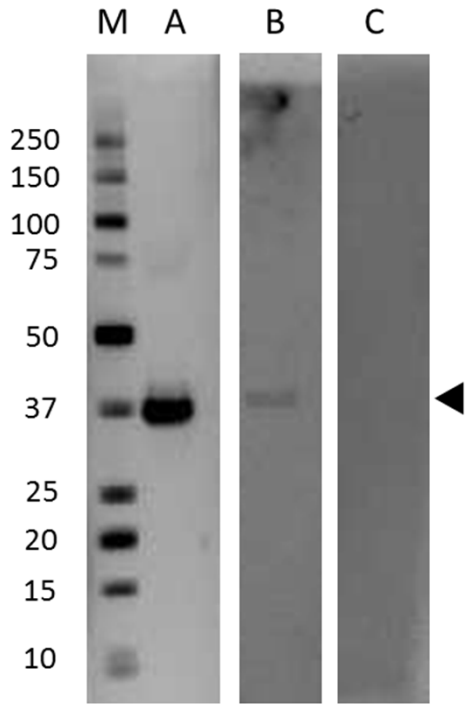
FACS histogram of Alexa 647-labeled ETX binding to cells transiently transfected with MAL expression vectors. ETX binds to cells expressing rMAL with greater affinity than to hMAL, whereas it fails to bind to CHO cells transiently transfected with constructs expressing zebrafish MAL, hMAL-L2F, or the GFP vector alone (overlapping curves at far left of the plot)

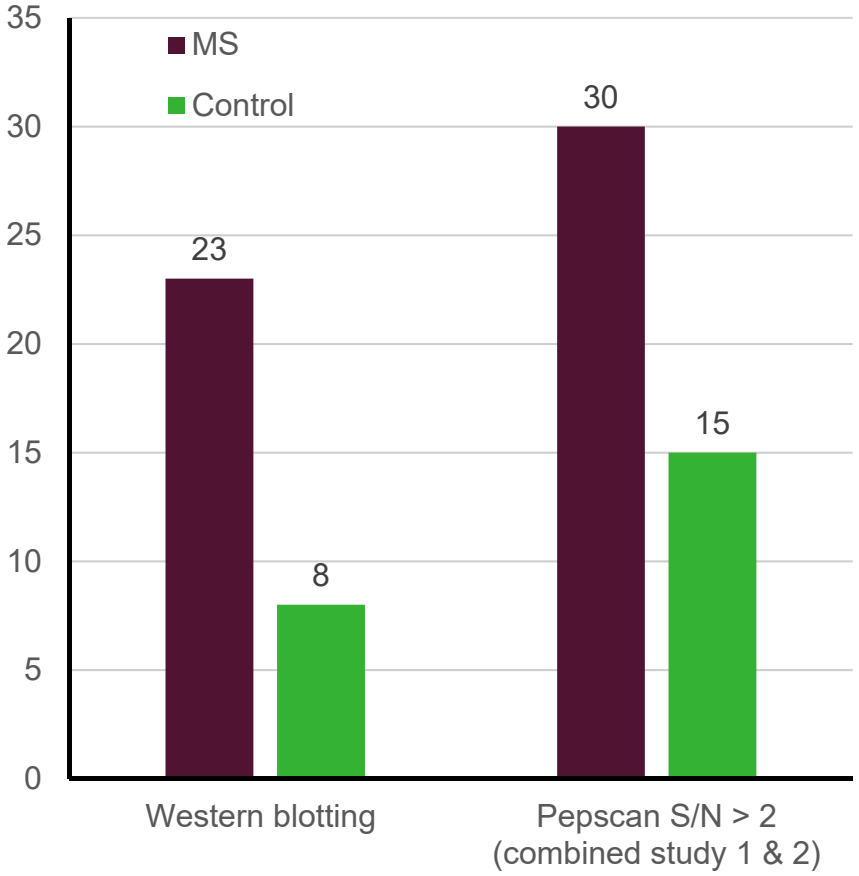


Rumah *et al*., 2015

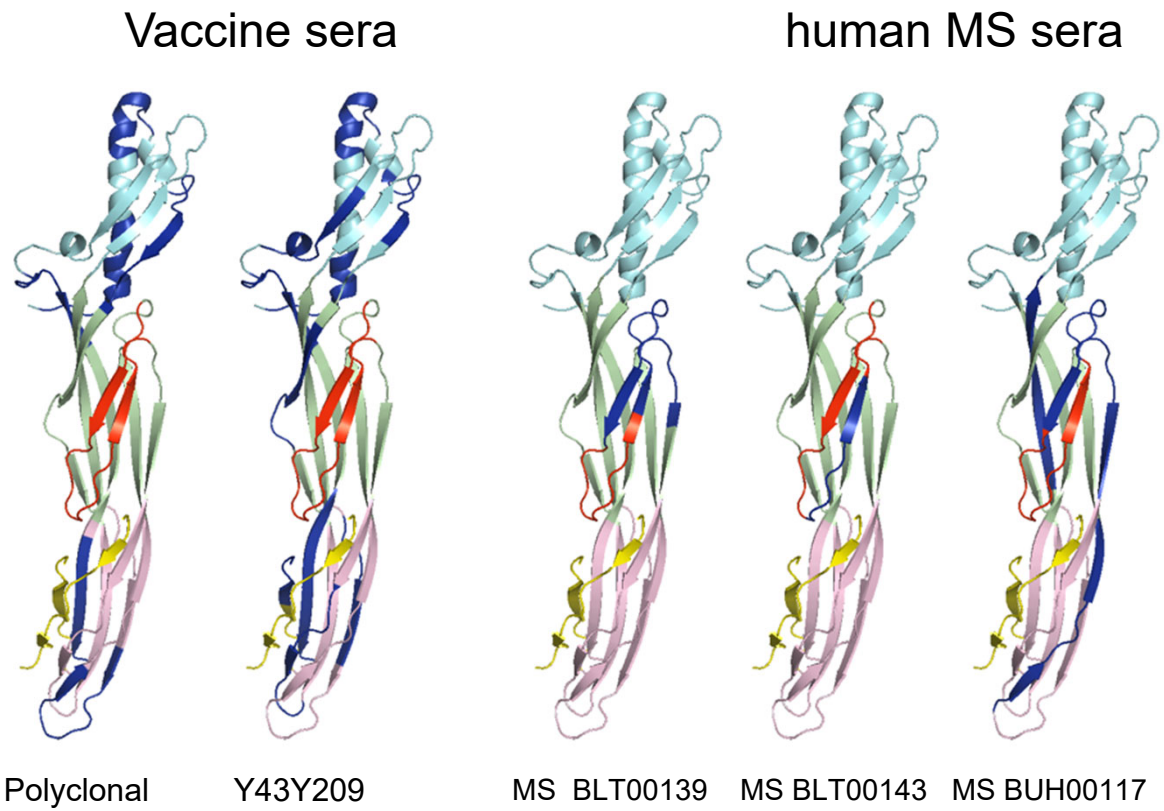
Antibodies to epsilon toxin in sera from MS patients detected using 2 different tests

- N= 64/64
- Excluding samples cross reactive with PA



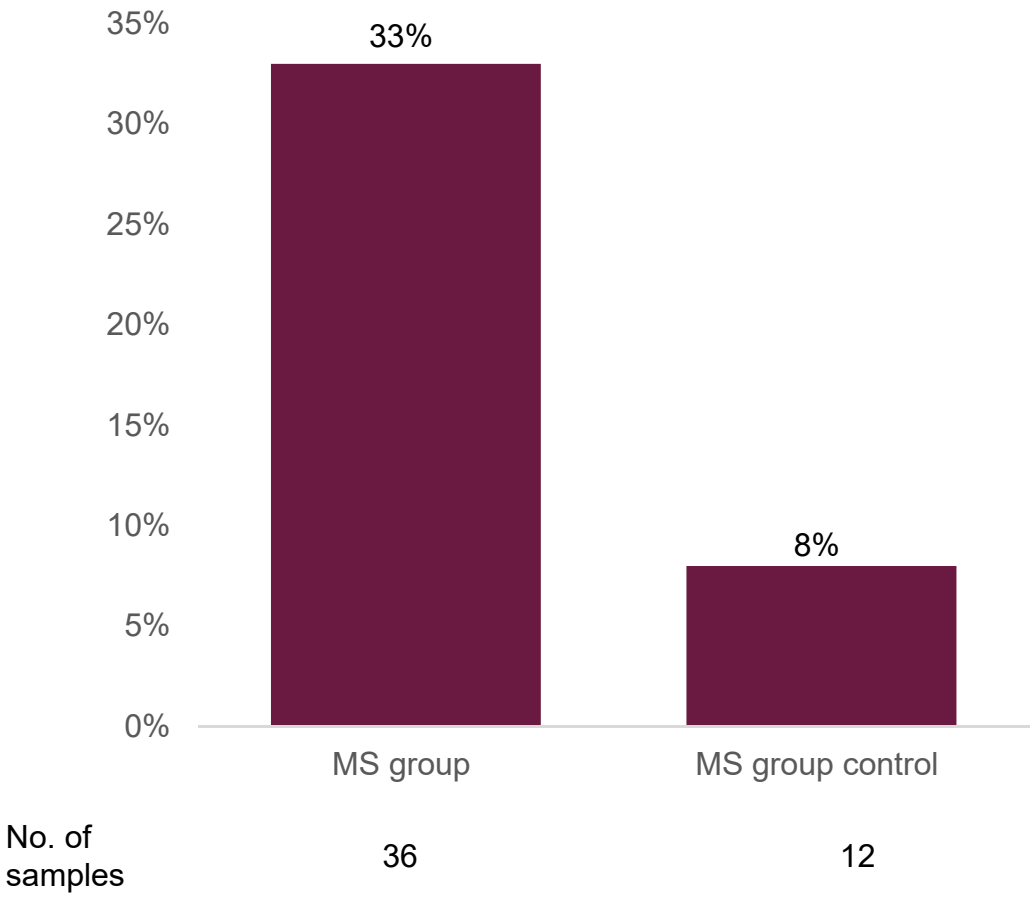


Antibody binding sites on epsilon toxin



- Epitopes recognised are shown in dark blue
- Antibodies in MS sera (BLT00139, BLT00143 & BUH00117) tend to recognise the loop that inserts to form the pore

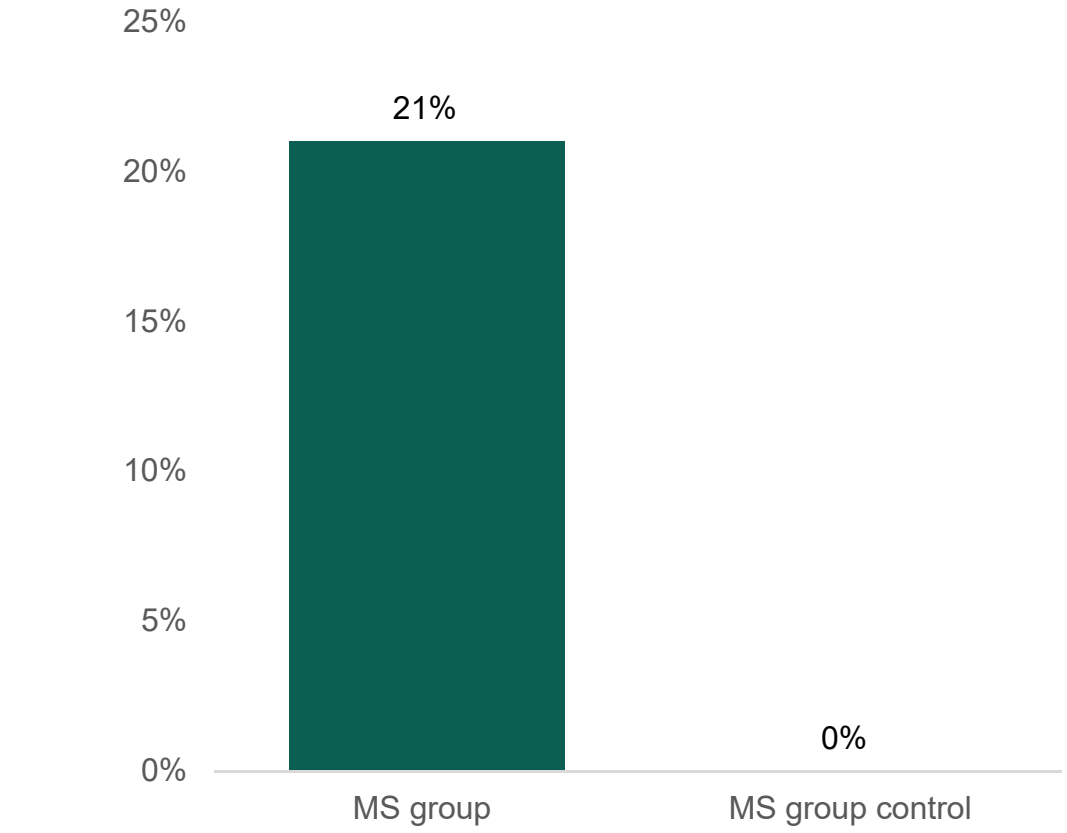
Epsilon toxin can be found bound to T-cells isolated from MS patients



ECTRIMS Poster (October 2018) by Jennifer Linden on epsilon toxin and CD4s and CD8s in MS patients entitled: "*Analysis of CD4+ cells reveal increased exposure of multiple sclerosis patients to clostridium perfringens epsilon toxin*"

Epsilon toxin detected on CD4+ cells of serum from various MS patients and controls. J Linden identified toxin in 12 of 36 MS patients (33%) compared with 1 in 12 controls (8%).

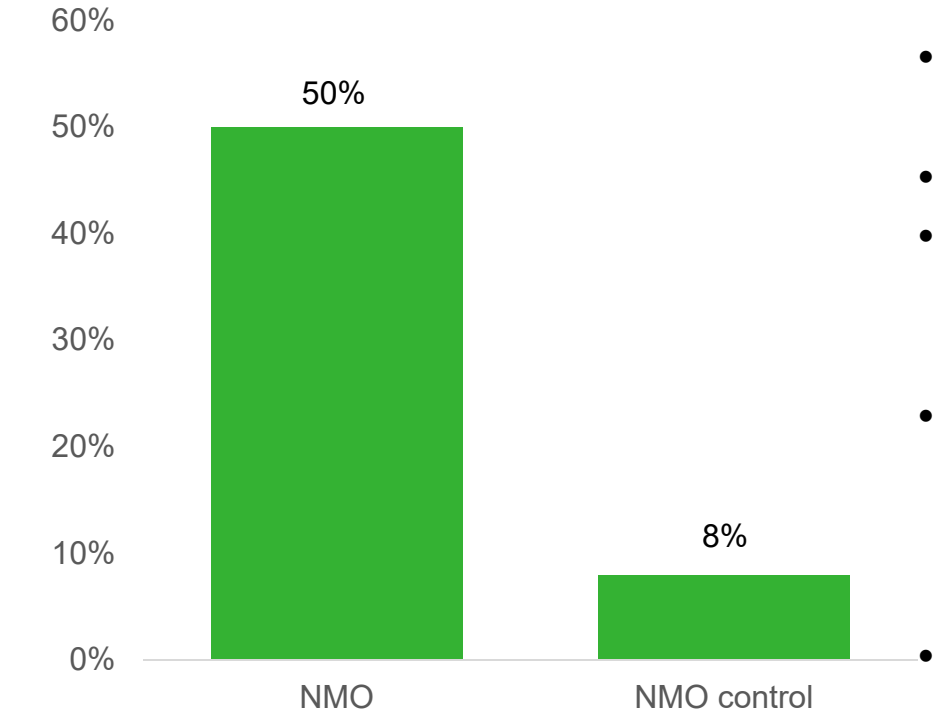
Strains of *C. perfringens* producing epsilon toxin are found in the gut of MS patients



ECTRIMS Poster by S Haigh showing C perfringens grown from faecal samples of MS patients but not controls entitled: *"Intestinal Colonization by Epsilon Toxin-producing Clostridium perfringens Strains is Associated with Multiple Sclerosis"*

C. perfringens (type B or D) cultured from 6 of 28 faecal samples from MS patients and zero bacteria were cultured from controls

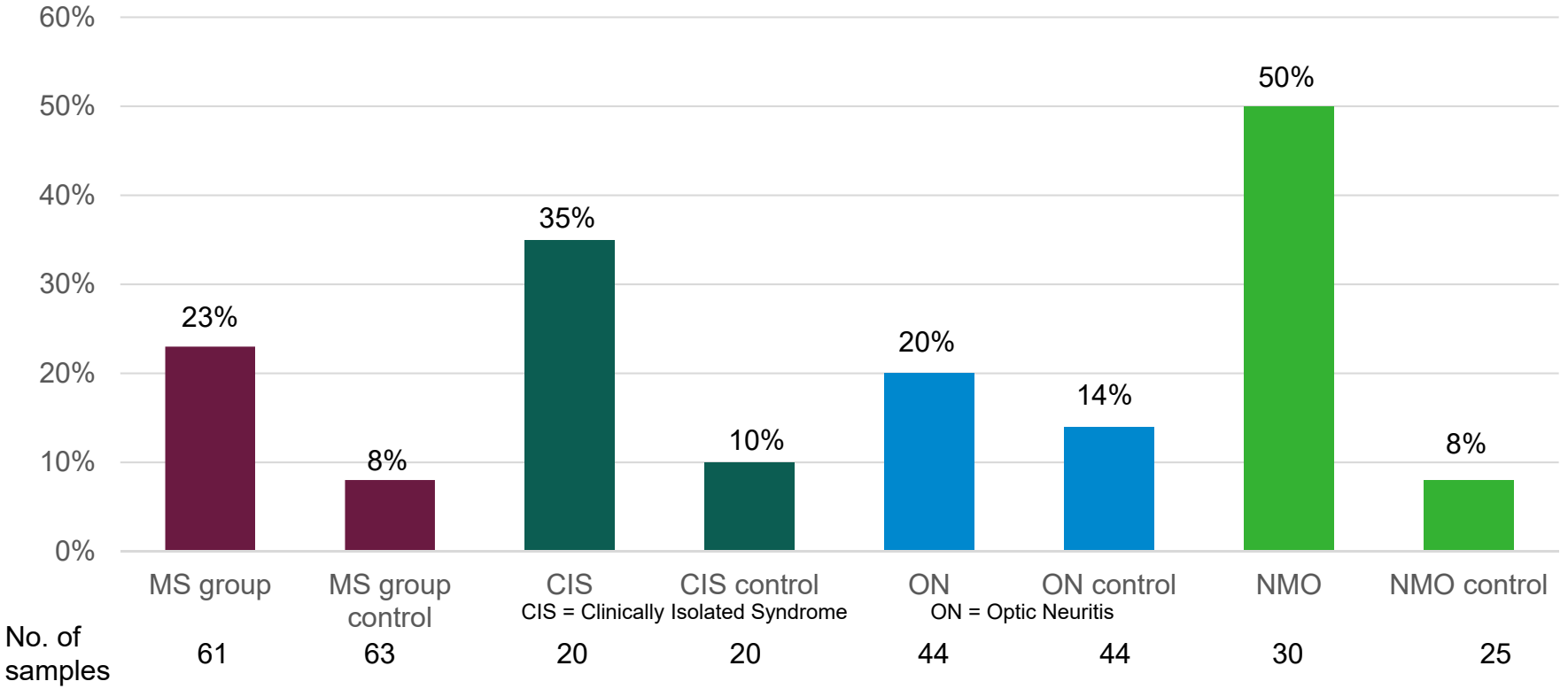
50% of NMO patients have serum antibody that reacts with epsilon toxin



- 30 sera from NMO patients from John Radcliffe Hospital, Oxford.
-
- All positive for antibodies to aquaporin 4.
- Age and gender matched controls from Exeter 10,000 study
- Screen using Western blotting

Source:
Pilot Study at Exeter University by Professor Titball

Comparison between NMO patients and MS patients (testing positive for antibodies to ETX)



- Using Western blotting, 24% of sera in the combined MS, CIS and ON groups reacted with Etx. In the control group, 10% of the samples reacted. Using Pepscan, 33% of sera tested reacted with at least one peptide, whereas in the control group only 16% of sera reacted. Out of 61 samples, 21 (43%) were positive to one or other testing methodology
- NMO patients on limited sample size showed double the rate (50%) of reactivity to ETX antibodies compared to the average for MS/CIS/ON patients (24%)

Evidence of Clostridium perfringens epsilon toxin associated with multiple sclerosis.
Sariqa Wagley, Monika Bokori-Brown, Helen Morcrette, more. Published: 21 April 2018.
https://journals.sagepub.com/doi/full/10.1177/13522458518767327

Proposed future research with GJCF

- Proposal to undertake a larger study
  - Up to 50 NMO patients
  - Longitudinal study – 4 samples per patient over say a 2 to 3 year period
  - Samples to be close to disease activity where possible
  - Source for controls? UK/GJCF
- Issues to be investigated
  - Do epsilon toxin antibody levels change over time?
  - Do episodes of disease relate to changes from epsilon toxin antibodies?
- Proposal to look for Epsilon Toxin in red blood cells from fresh blood samples from NMO patients
  - Similar research is currently being undertaken by K. Rumah with MS patients
- Propose using sensitive reagents from Tim Vartanian labs
- Possible multi-centre testing through GJCF contacts?