VAC2VAC Vaccine batch to vaccine batch comparison by consistency testing

Experience in extending the approach of cell-based TCP and MLD assays to *Clostridium perfringens* vaccines

Arjen Sloots
Intravacc, Bilthoven, The Netherlands

Novel *in-vitro* models as alternative to in-vivo toxoid vaccines testing: *Clostridium septicum* vaccine as proof of concept
Virtual Workshop, 9 & 10 March 2021

Main objective within VAC2VAC project

- Contribute to the development of *in vitro* assays addressing specific toxicity of ***Clostridium perfringens*, type C (non-inactivated) antigen** as an alternative to the currently used *in vivo* mouse tests.

Approach

- Use the same principle as described for *in vitro* toxicity testing of *C. septicum* vaccines. However, VERO cells are only poorly susceptible to the β-toxin of *C. perfringens* type C, considered the main antigen of this vaccine.
- **Hence, main goals defined as follows:**

  1. Identification of cell line specifically susceptible to the *C. perfringens,* type C β-toxin.

  2. In case of identification of such a cell line, show feasibility of assay development based on this cell line for assessment of *C. perfringens* C β-toxin activity *in vitro*.

  - Start with cell-based alternative to MLD in mice using ***C. perfringens* C non-inactivated antigen**
  - If successful, continue with development of cell-based assay (CBA) for assessment of residual toxicity of inactivated (toxoid) antigen

First steps

- Literature search for β-toxin-sensitive cell lines yielded the human HL-60 and THP-1 cell lines. In addition, one of our VAC2VAC industry partners recommended testing the rat A10 cell line.
- Parallel approach: Transfect VERO cells with P2X7 receptor, published to be the receptor involved in β-toxin activity (Nagahama *et al.*, 2015) to render the cells sensitive to β-toxin.

Initial results:

- HL-60 proved very difficult to culture and stable VERO-P2X7 transfectants did not show enhanced susceptibility to *C. perfringens* C non-inactivated antigen compared with parental VERO cells (Crystal Violet staining). →**HL-60 & VERO-P2X7 not tested further!**
- In contrast, **Rat A10 and human THP-1** showed β-toxin-induced toxicity in a concentration dependent manner using the MTS assay for THP-1 cells and Crystal Violet staining for the A10 cells.

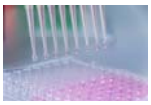
Next step

- Use of neutralizing Mab 10A2 against β-toxin and international anti-β-toxin standard to determine whether observed toxicity on **A10 and THP-1** cell lines is β-toxin-specific

  - Crude *C. perfringens* C non-inactivated antigen used from one of our VAC2VAC industry partners in all experiments (also called 'end-of-fermentation supernatant'; contains β-toxin)
  - Mab 10A2 purchased from USDA
  - International anti-β-toxin standard (CPBETAAT) purchased from NIBSC
  - Equal volumes of diluted non-inactivated antigen were pre-incubated with dilutions of antitoxin in medium, followed by 30 min incubation at 4°C on an orbital shaker (250 rpm).

Basic assay set-up

A10 cell line:



37°C, 5% CO_2 overnight

37°C, 5% CO_2 16-24h

Seed cells in 96-well plate (10,000/well) — **Day 1**

Add toxin — **Day 2**

Assess viability/toxicity: Crystal Violet staining — **Day 3**

THP-1 cell line:

37°C, 5% CO_2 overnight

37°C, 5% CO_2 16-24h

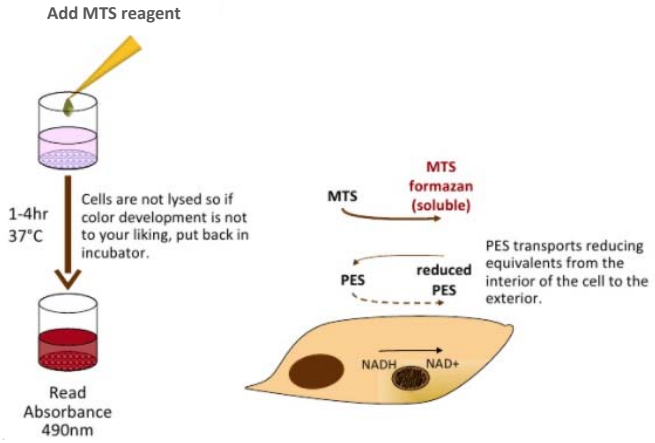
Seed cells in 96-well plate (25,000/well) , add PMA (300 ng/ml) — **Day 1**

Add toxin — **Day 2**

Assess viability/toxicity: MTS assay — **Day 3**

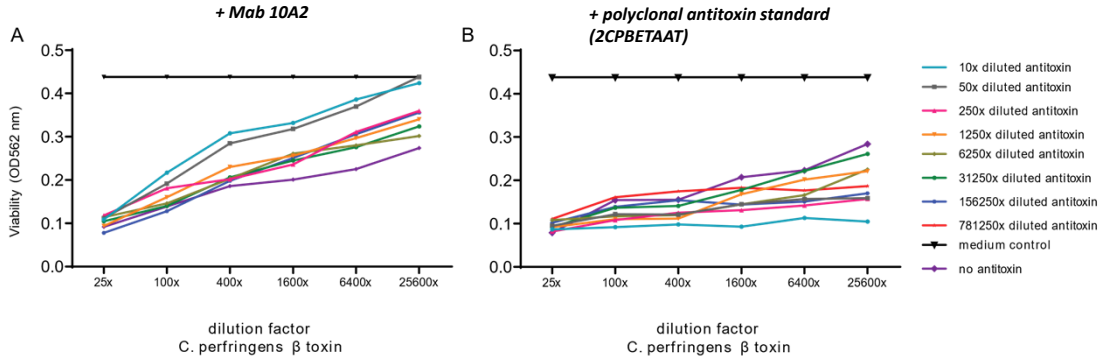
MTS assay as a read-out for cell viability

- Assay is based on bio-reduction of MTS by living cells into a soluble colored formazan product.
- The amount of formazan produced can be quantified by reading absorbance at 490nm.



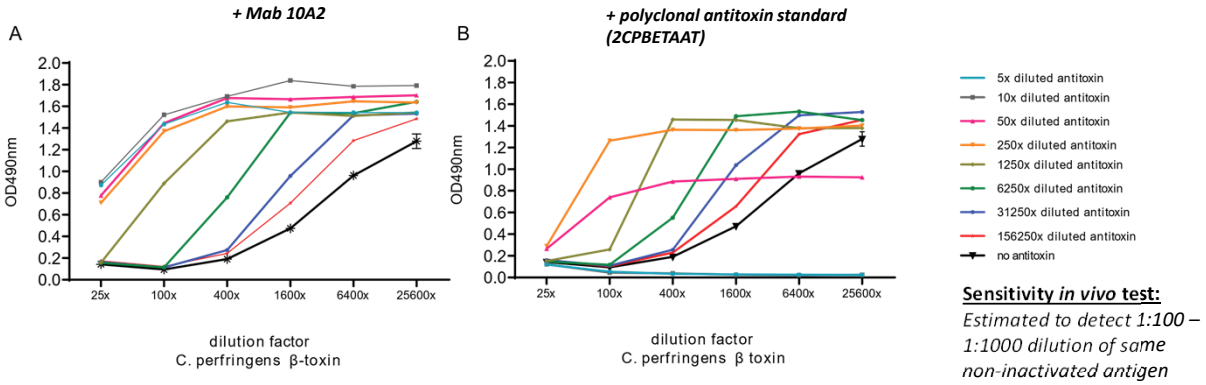
Toxicity of *C. perfringens* C non-inactivated antigen on A10 cells is only partially β-toxin dependent

- *<u>Readout:</u> Crystal Violet staining*



Toxicity of *C. perfringens* C non-inactivated antigen on THP-1 cells is β-toxin specific

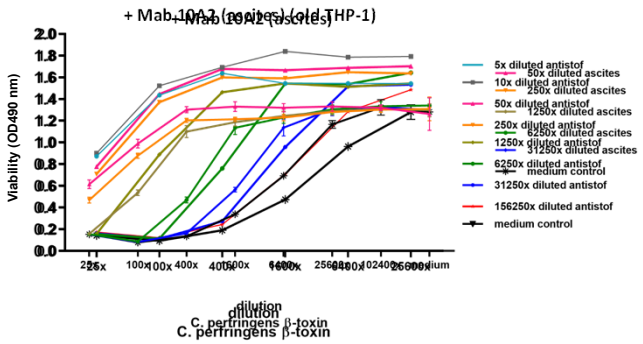
- *Readout: MTS assay*



Sensitivity *in vivo* test:
Estimated to detect 1:100 – 1:1000 dilution of same non-inactivated antigen material (VAC2VAC Industry partners, personal communication) [9]

Results are reproducible with newly purchased THP-1 cells (ATCC: TIB-202)

- *PMA-treated THP-1 cells exposed to dilutions of C. perfringens non-inactivated antigen, either pre-incubated with anti-toxin (Mab 10A2) or not. Readout: MTS assay (in triplicate)*

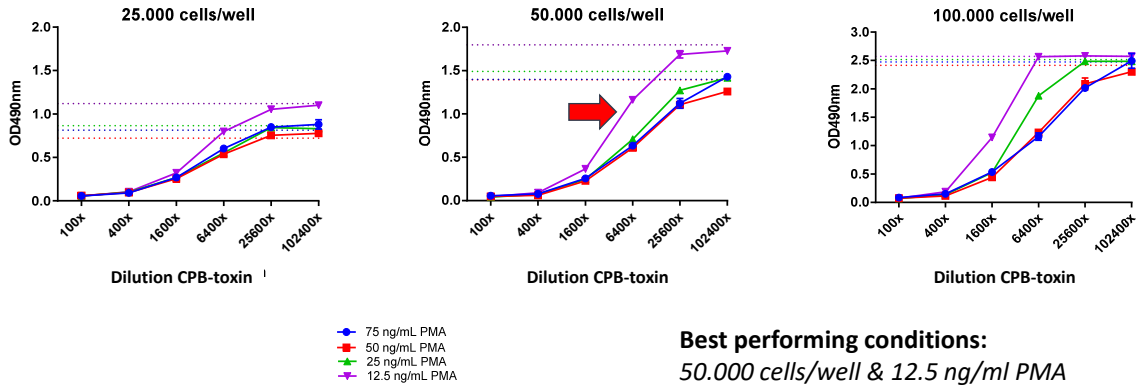


Assay optimization - Cell density, PMA concentration & FBS amount

- Experimental set up used thus far:
  - 25.000 cells/well (96-well plates)
  - 300 ng/ml PMA
  - 10% FBS

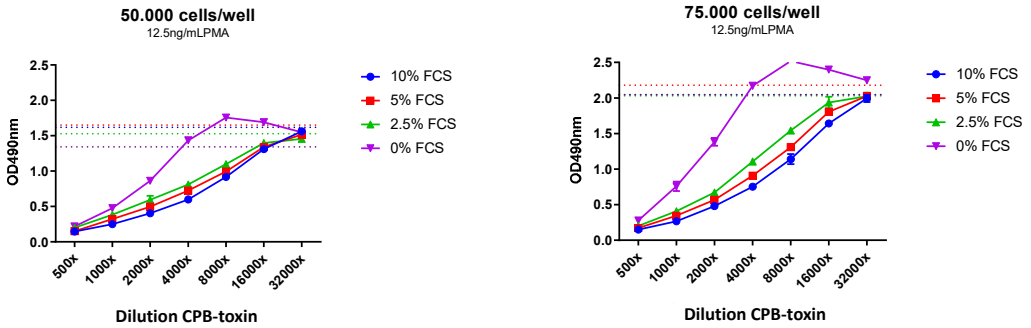
- First optimization experiments showed that **50.000 cells/well** performed better than 25.000 or 100.000 cells/well (using 300 ng/ml PMA & 10% FBS; not shown).
- In addition, **75 ng/ml PMA** performed better than 150 or 300 ng/ml (using 25.000 cells/well & 10% FBS; not shown).

Assay optimization - Cell density & PMA concentration



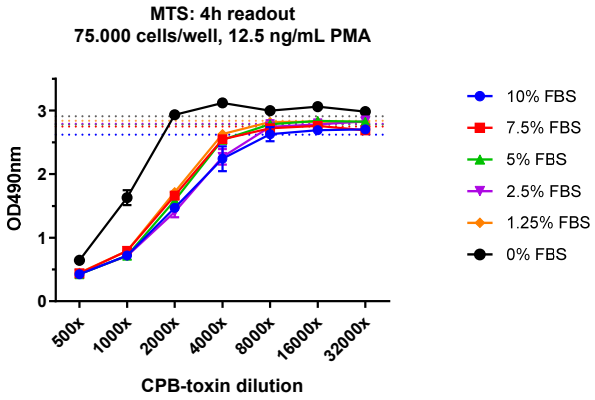
Best performing conditions:
50.000 cells/well & 12.5 ng/ml PMA

Assay optimization - Cell density & FBS amount



Best performing conditions:
75.000 cells/well & 12.5 ng/ml PMA
2.5, 5 or 10% FBS does not seem to make a large difference

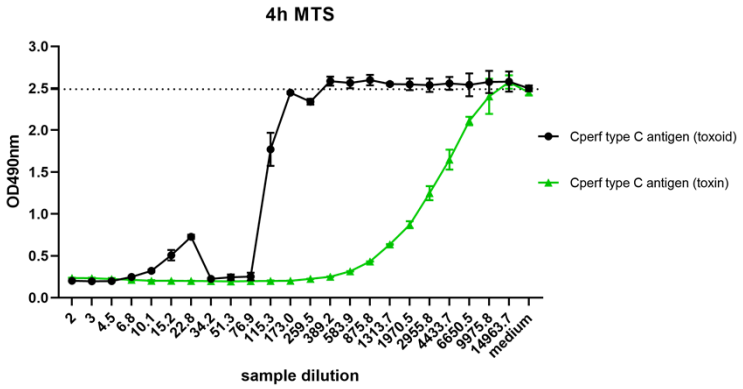
Assay optimization - FBS amount



Conclusions:
1.25% - 10% FBS has only minor influence on the assay.

0% FBS: Curve reaches plateau already at relatively low dilutions. Suggests that assay has less discriminatory power when no FBS is used.

Further development THP-1 CBA to assess toxicity of *C. perfringens* C inactivated antigen (toxoid)



- Dotted line represents the average absorbance reading of untreated control wells
- 1.5-fold dilution series

Conclusions (I)

- A10 cells are susceptible to *C. perfringens* type C non-inactivated antigen but observed toxicity is not β-toxin specific
- PMA-treated THP-1 cells are specifically susceptible to the β-toxin that is present in the *C. perfringens* type C non-inactivated antigen
- Results with the 'in-house' THP-1 cells can be reproduced with 'new' THP-1 cells ordered at ATCC
- Optimized experimental setup includes 75,000 cells per well (96-well plate) and 12.5 ng/ml PMA, in which the amount of FBS can be anywhere between 1.25% and 10%
  - ➢ *An optimized protocol for the THP-1-based MLD assay has been prepared and transferred to the industry partners for further assessment and validation*

Conclusions (II)

- **THP-1 CBA may be suitable for assessment of residual toxicity of inactivated (toxoided) antigen preparations. However, there is a problem to solve:**
  - **The inactivated antigen still contains residual formaldehyde. This makes it difficult to determine whether the cell death observed at low dilutions is caused by residual β-toxin or by formaldehyde (or both).**

➢ **<u>Next step:</u> Repeat the THP-1 CBA with inactivated antigen in the presence of formaldehyde-neutralizing agents**

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