GENERAL TOXICITY TEST ON CLOSTRIDIALS

CELL-BASED TOXICITY ASSAYS FOR CLOSTRIDIAL ANTIGENS

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For:

3Rs implementation in veterinary vaccine batch-release testing: Current state-of-the-art and future opportunities

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MSD Animal Health

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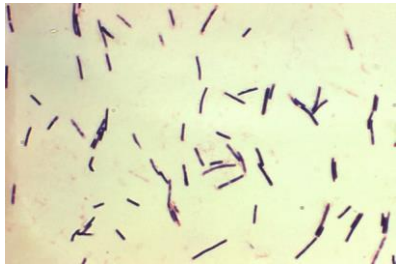
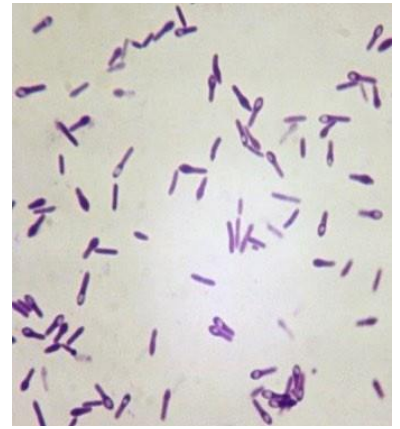
Introduction – Clostridial antigens and vaccines

- *Clostridial* bacteria are ubiquitously found in soil and the intestines of animals.
- Infection can be triggered by a variety of conditions ranging from a change in diet through to shearing cuts.
- Toxins produced by clostridia are typically responsible for pathology.
- Most clostridial toxins are pore forming, creating perforations in cell membranes, leading to cell death.
- Disease progression is usually **rapid** making treatment with antibiotics impractical.
- Vaccination is the best strategy.





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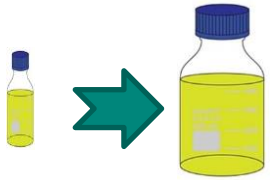
Introduction – High level manufacturing process

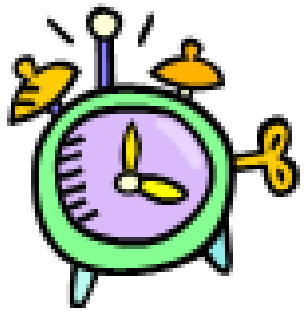


Initial cultures

Fermentations

Centrifugation for bacterial cell removal

Addition of formaldehyde to supernatant containing toxin



Incubation until toxin has been converted into toxoid

Antigen testing methods

Vaccine formulation

Vaccine testing methods

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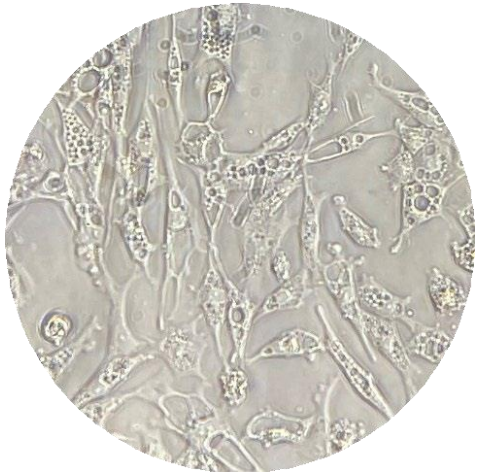
Introduction – Cell line cytotoxicity assays

- Cell viability and proliferation are key indicators of cell health
- Physical and chemical stress can affect cell viability and metabolism
- Cytotoxicity can be caused via various mechanisms:
  - Cell membrane damage
  - Seizing protein synthesis
  - Irreversible receptor binding
  - Inhibition of biochemical reactions within the cell.
- Cell-based *in vitro* assays can be developed to quantify these changes



Review: Sultan Aslanturk (2018) *in vitro* cytotoxicity and cell viability assays: Principles, advantages, and disadvantages, *IntechOpen.*

Scope of the project

Overall aim:

To develop, validate and implement toxicity assays for QC testing of *Clostridium* antigens.

***C. perfringens D*, *C. chauvoei*, *C. septicum* and *C. novyi* antigens**

Project milestones

- Optimise cell line assay for *C. perfringens* type D antigens
- Identify a suitable cell line for toxicity testing of *C. chauvoei* antigens
- Confirm sensitivity of Vero cell line to *C. novyi* and *C. septicum antigens*
- Submit dossier variation to regulatory authority

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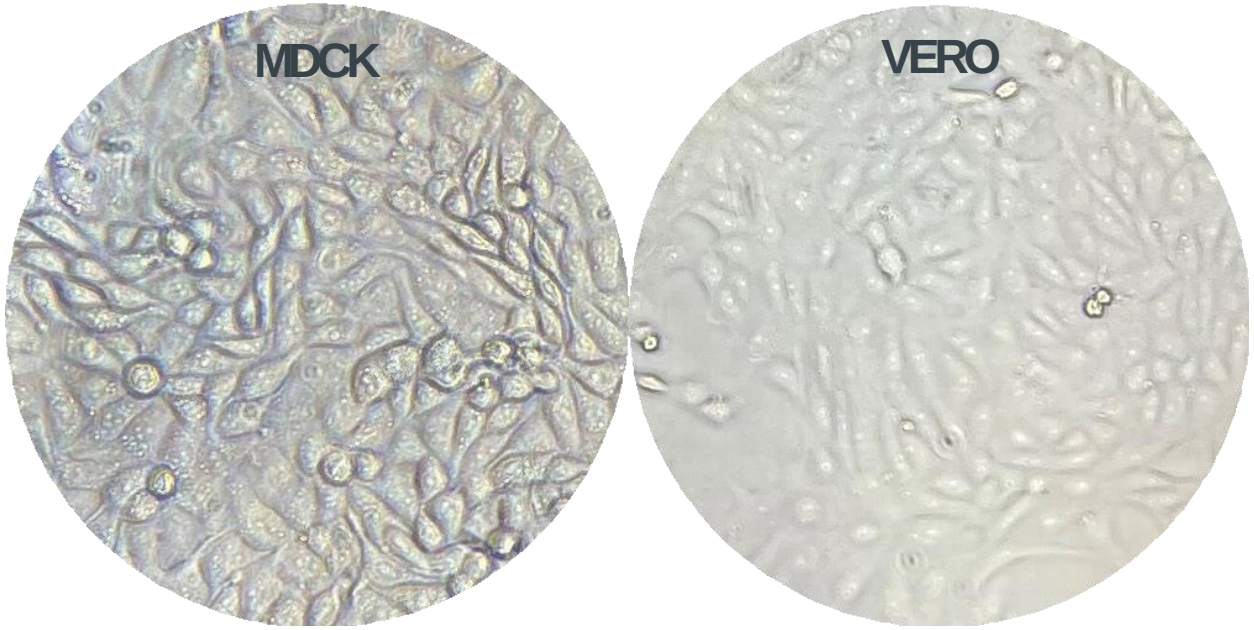
Cell lines

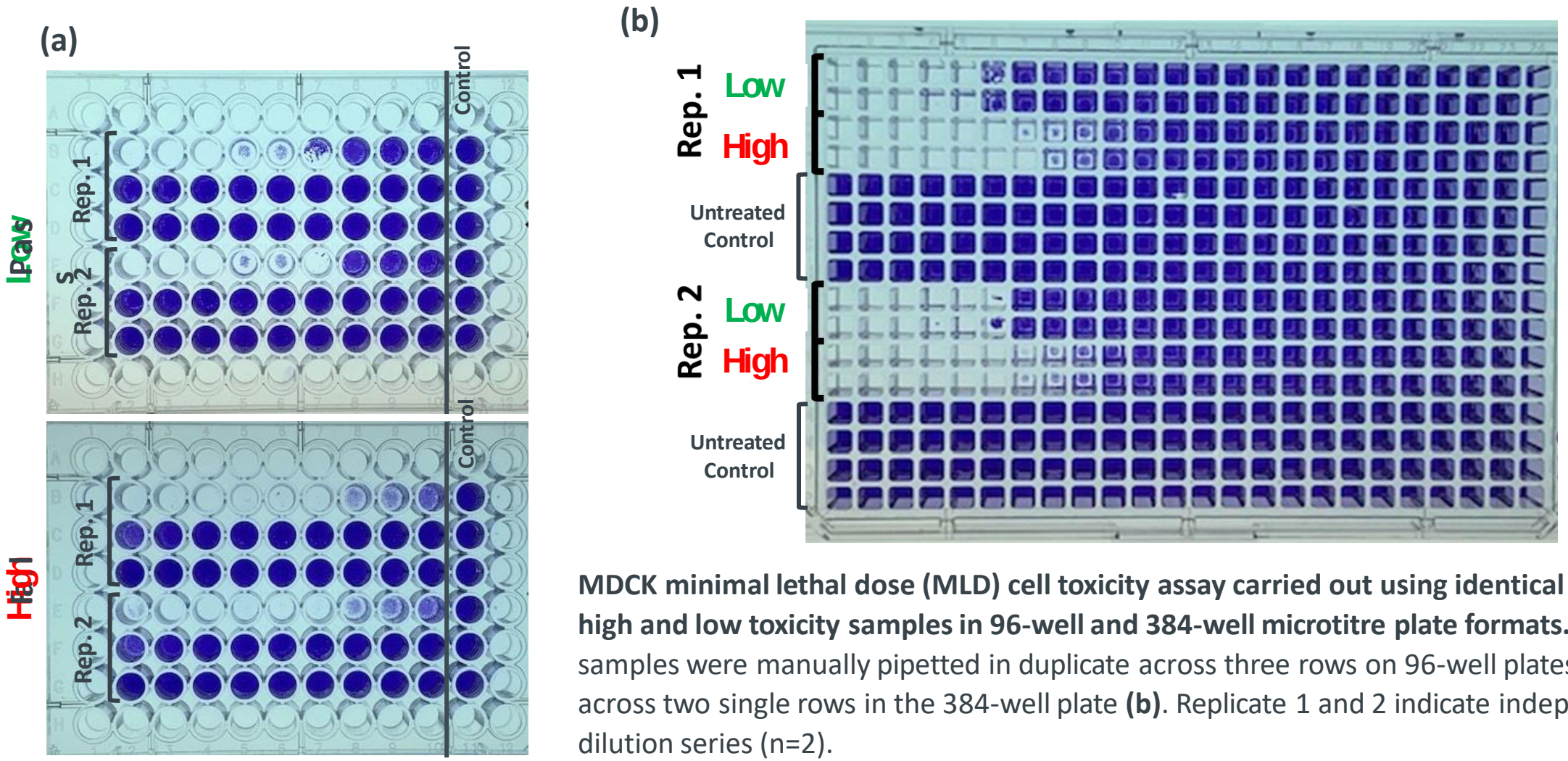
Strain	Cell line
Clostridium perfringens D	MDCK
Clostridium chauvoei (bacterin)	MDCK
Clostridium chauvoei (toxoid)	MDCK
Clostridium septicum	Vero
Clostridium novyi	Vero



MDCK: Madin-Darby canine kidney

Vero: African green monkey kidney epithelial cells.

C.perf. D MLD assay demonstrates equivalence across both microtitre plate formats.



MDCK minimal lethal dose (MLD) cell toxicity assay carried out using identical CpD toxoid high and low toxicity samples in 96-well and 384-well microtitre plate formats. The samples were manually pipetted in duplicate across three rows on 96-well plates **(a)**, and across two single rows in the 384-well plate **(b)**. Replicate 1 and 2 indicate independent dilution series (n=2).

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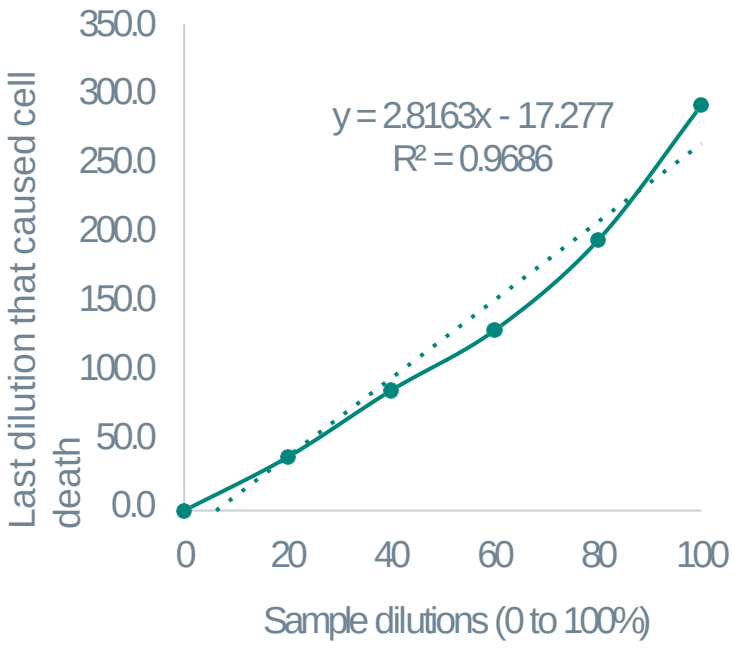
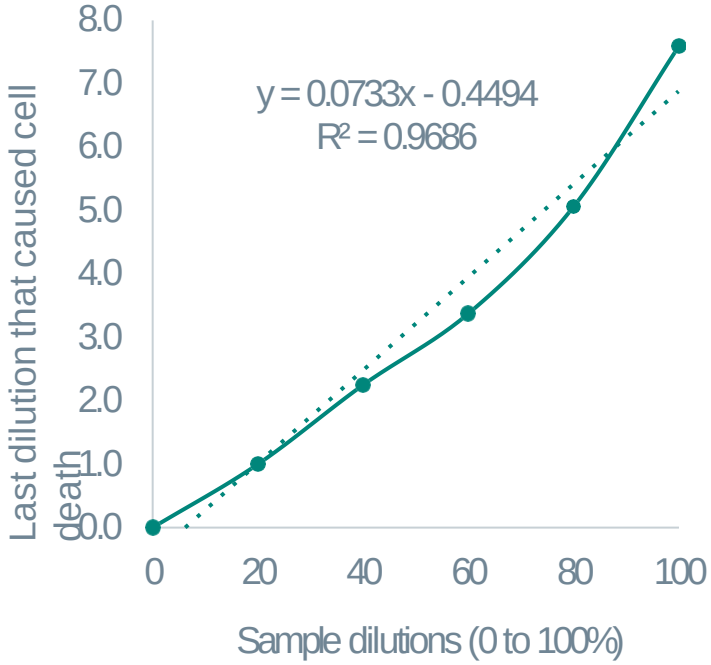
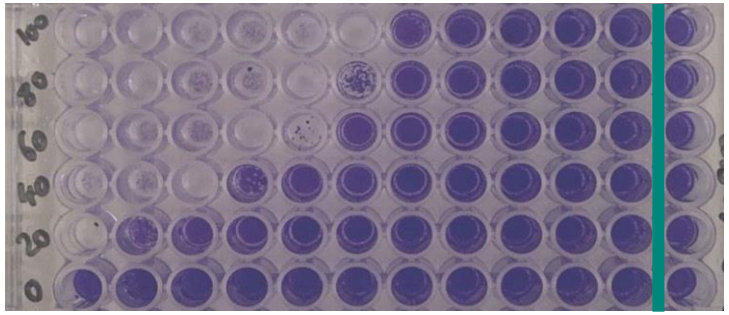
Both MDCK and Vero cell line assays demonstrate linearity



C. septicum
(Vero assay)



C. perfringens D
(MDCK assay)



Assay linearity was demonstrated for both cell lines through the dilution of toxoid samples

Validation studies: Robustness, repeatability and intermediate precision

Robustness parameters assessed:

- Overnight incubation time (**15h**, **18h** and **21h**)
- Cell seeding density (**1 x10^5**, **3 x10^5** and **5 x10^5** cells/ml)
- Cell refrigeration (4°C) time post-detachment (**fresh cells**, **2 days** and **4 days old)**

Repeatability and intermediate precision studies

- Performed in duplicate on three independent days by two operators using high, medium and low toxicity samples.
  - Low, medium and high sample toxicity was determined through pilot toxicity assays.
  - Samples were diluted or spiked with toxin in an attempt to reduce or increase toxicity, respectively.

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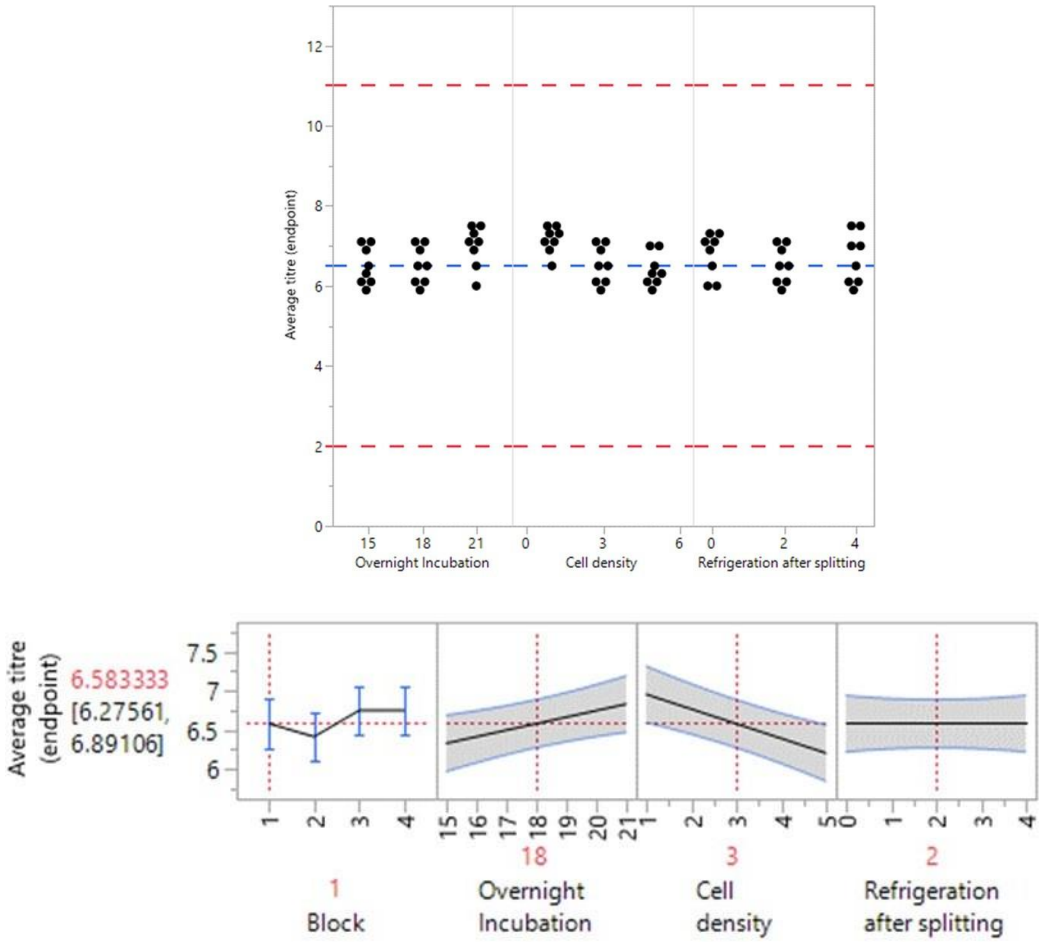
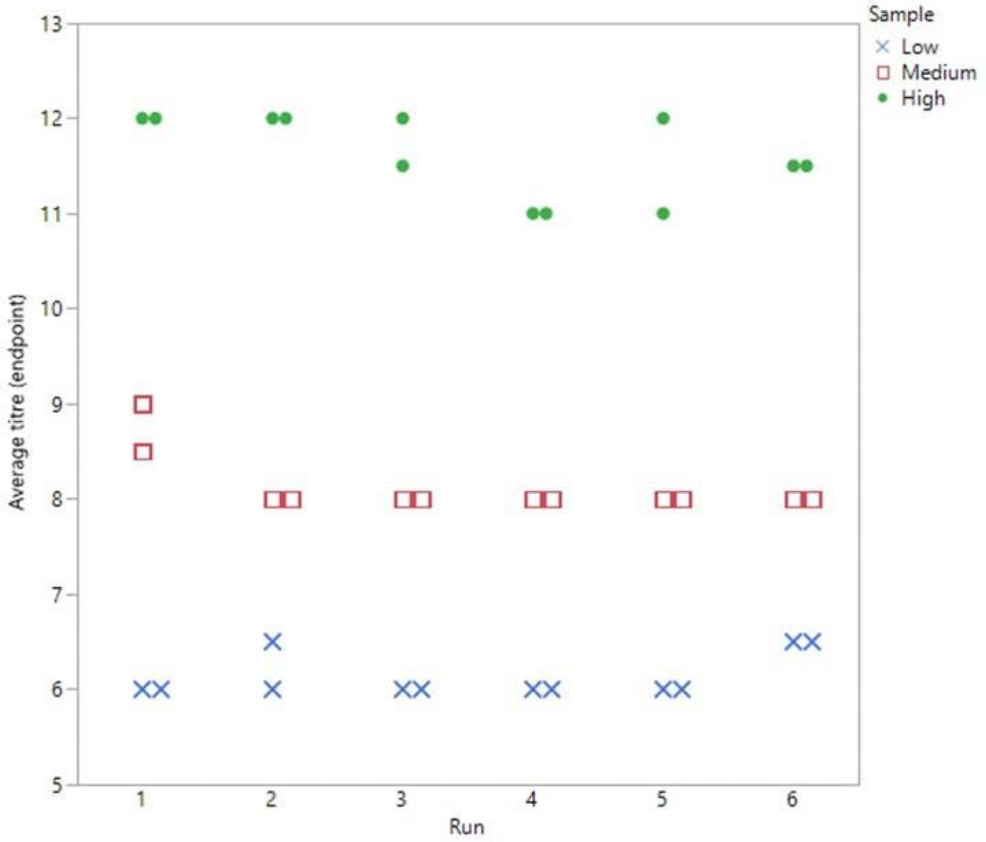
C. perfringens D validation studies: Robustness and intermediate precision



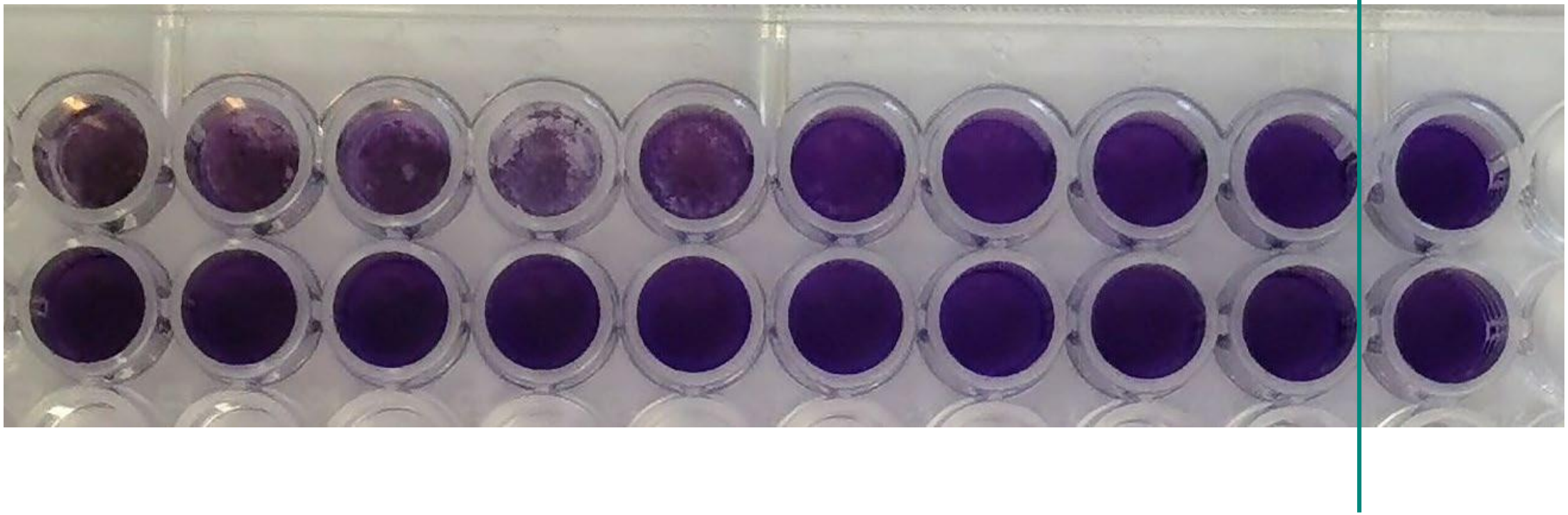
Robustness



Precision

Technical challenges: Alleviating the impact of formaldehyde on cell monolayers

- The formaldehyde content of toxoid samples lead to false positive staining of initial wells containing highest concentrations of sample.
- *C. Perf. D* toxicity assay was previously abandoned from QC due to these non-specific readings.



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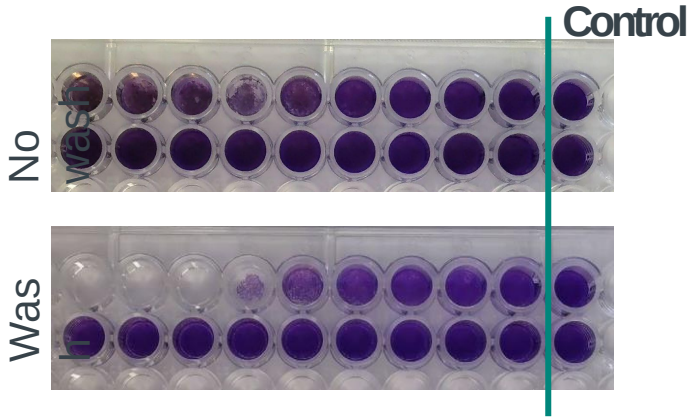
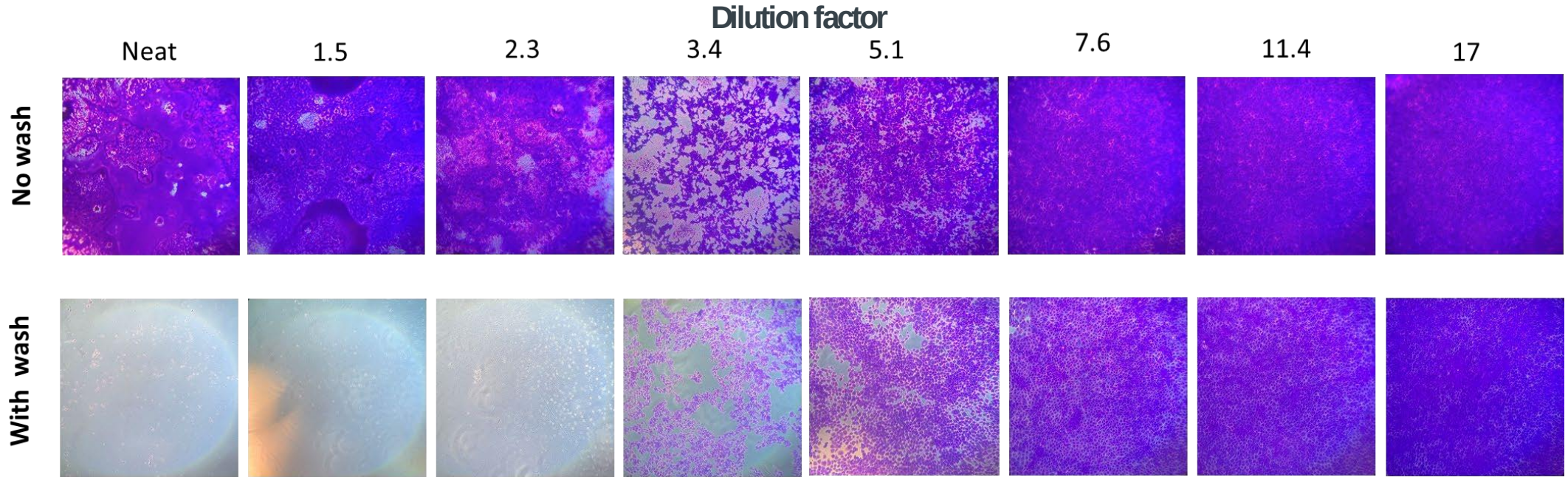
Technical challenges: Alleviating the impact of formaldehyde on cell monolayers

Solution: The inclusion of a pre-stain wash step in tap water completely removes false positive that appear within initial wells.





Characterisation of cell line sensitivity towards *C. chauvoei* antigens

- A sensitive cell line for *C. chauvoei* had <u>not yet been described.</u>

<u>Aims:</u>

- Screen and compare the suitability of several cell lines for use with *C. chauvoei* cell-based toxicity assays.
- Assess which cell line is most suitable for bacterin and toxoid samples.
  - Both are used as components of the vaccine.

MDCK cell line demonstrates toxicity to *C. chauvoei* antigens

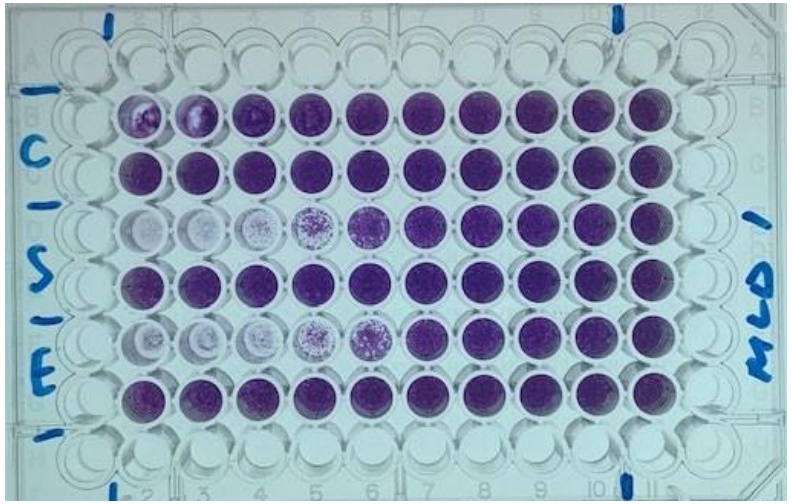
	Samples from manufacturing to estimate toxicity of chauvoei antigens on cell lines (active toxin)			
	End of fermentation	Cell pellet	Supernatant	Supernatant Conc.
MDCK	7	3	6	9
VERO	4 – 5	2	5 – 6	-

Simulated concentration of QC samples

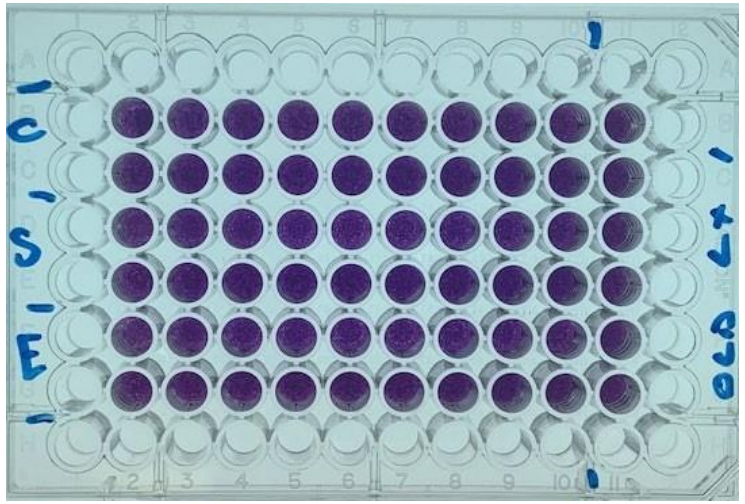
Sensitivity of cell lines to *C. chauvoei* antigens

We should aim for a cell line that is sensitive in order to detect any residual toxin in the toxicity samples.

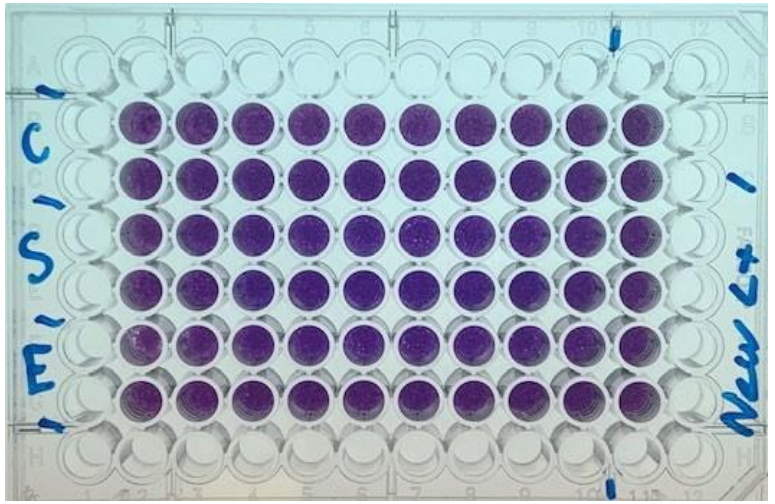
Neutralisation of toxicity using antisera



Fermentation samples MLD



Fermentation samples L+
Antisera A (from multivalent vaccine)



Fermentation samples L+
Antisera B (from monovalent C. chauvoei vaccine)

Both antisera (diluted 1:50) effectively neutralised sample toxicity.

Note: Fermentation samples are not concentrated to production levels.

Conclusions

- All cells line assays showed good linearity with toxin concentration
- All four cell line assays were found to be robust, reproducible and precise
- The presence of formaldehyde in the test samples that caused problems was remediated with a pre-stain wash step
- 3 of the tests described have been approved by the European Regulatory authorities
- Awaiting feedback for 2 tests

Ongoing work

- Transfer of new *in vitro* tests to Quality Control laboratories

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THANK YOU

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Assay Overview