Sensitivity of an alternative format and readout for an *in vitro* infectivity assay for porcine circovirus (PCV)

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Outline

- Background
  - Driver for a novel infectivity assay
- Methods
  - Novel method using PK-15 cells, 3 day splits, QPCR readout
  - Comparison to an adaptation of a “9 CFR 113-like” method using PT-1 cells, 7-day splits, and immunofluorescence (IF) readout
- Results
  - Sensitivity and repeatability of novel method
  - Comparison to “9 CFR 113-like” method
- Next steps

Why pursue PCV infectivity methods?

- QPCR and short amplicon endpoint PCR detected fragments of nuclease-resistant PCV-2 DNA
- Our longer amplicon endpoint PCR did not detect PCV DNA - **but we did not know this when our investigation began**
- Infectivity methods were not yet fully validated or widely available commercially
- Needed to reexamine infectivity methods in event of positives in long amplicon PCR (false or otherwise)
- Should apply best methods to support raw material risk assessment / viral inactivation

Detecting infectious PCV *in vitro*, ~May 2010

- 1987 – PCV replication dependent on enzymes present during S-phase of cell cycle (Tischer *et al* Arch Virol 96:39-57), implying that contact-inhibited cells might not support as robust viral amplification as cells actively growing
- PK-15 cells widely used to detect PCV replication
- Merck had access to a non-validated commercial assay using PT cells, 1-week splits, and IF detection
- FDA reported using ST cells, 3-day splits, and nucleic acid detection (VRBAC, 07May2010)
  - ***take-home lesson: infectivity might be masked if cultivation and detection conditions were not optimal.***

Initial steps

- Explore novel assay based on FDA method description, but using PK-15 cells
  - Sensitivity, reproducibility
  - Compare with a “9 CFR 113-like” assay
- Validation of existing PCV IF-based assay at commercial lab was also underway

“9 CFR 113-like” Assay

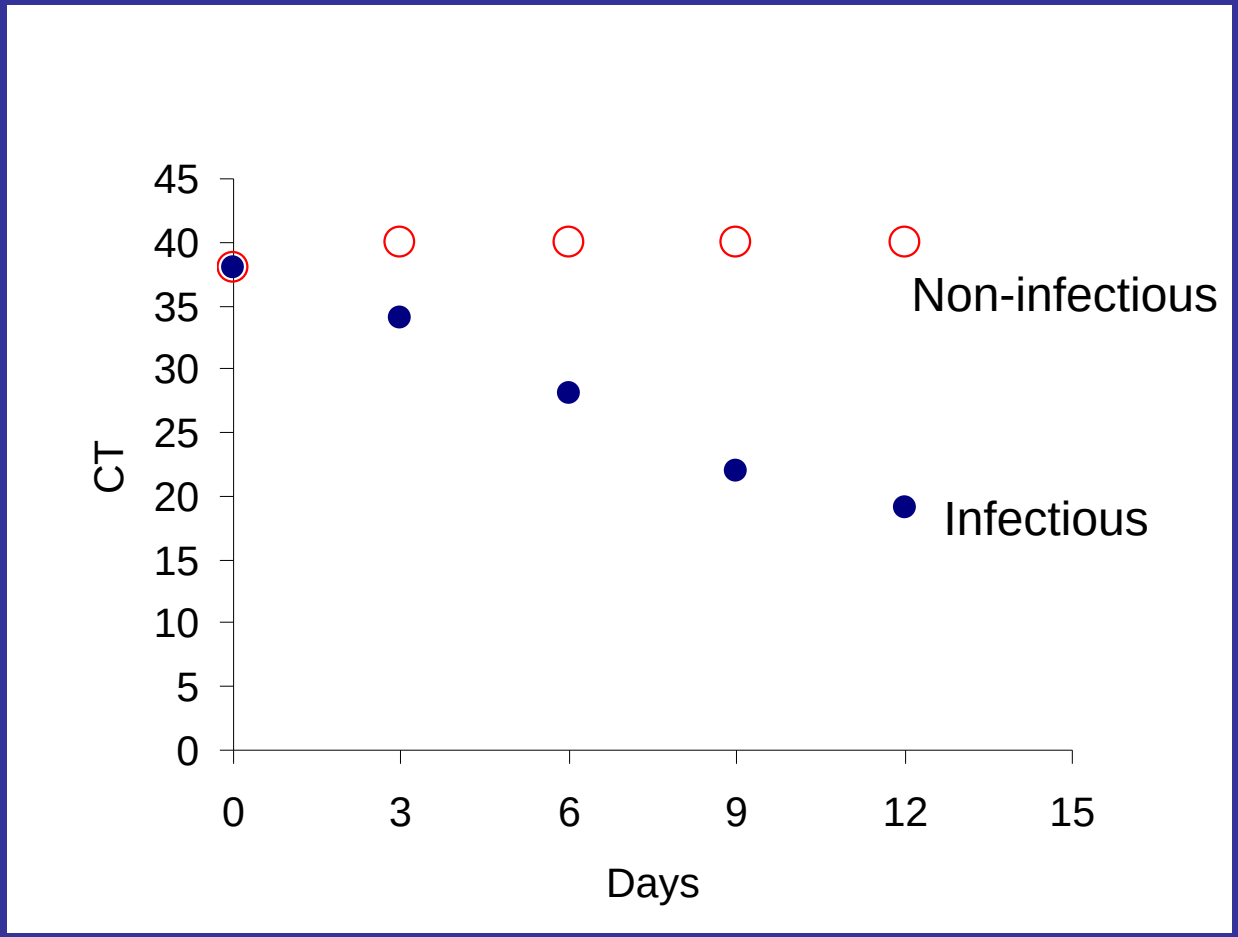
- Specific testing for bovine and porcine viruses for animal derived reagents to comply with 9 CFR 113.53 (culture & infection) and 113.47 (IFA)
- 21-day test, subcultures in Vero and one other species-specific cell line at days 7 and 14.
- Monitor for CPE throughout, hemadsorbing viruses at day 21, and specific viruses by IFA at day 21
- 9 CFR 113 does not specify testing for PCV,
  - so corresponding assay is described here as “9 CFR 113-like”

Methods

PK-15 cells, 3-day splits, QPCR

- Pre-infection
  - Plant T-75 flasks 24 hours prior to infection such that cells are subconfluent at infection
- Infection
  - Remove medium, rinse
  - Add 3-6 mL infection medium/flask with diluted standards or test article
  - Incubate 60-90 min 37oC, 5% CO2
  - Remove inoculum, rinse
  - Refeed
- Post-infection
  - After 3 days,
    - remove culture medium for PCV analysis
    - Remove cells using recombinant trypsin, plant half the cells in a new T-75 flask, freeze the remaining half of cells
  - Repeat for up to 9 splits
- QPCR
  - Total nucleic acid extractions using Qiagen Viral RNA kit
  - Real time QPCR using primers/probe recognizing both PCV-1 and PCV-2

What is expected?



PT-1 cells, 7-day splits, IFA

- Pre-infection
  - Plant T-75 flasks 24 hours prior to infection such that cells are subconfluent at infection
- Infection
  - Remove medium, rinse
  - Add 3-6 mL infection medium/flask with diluted standards or test article
  - Incubate 90 min 37oC, 5% CO2
  - Remove inoculum, rinse
  - Refeed
- Post-infection
  - After 7 days,
    - remove culture medium
    - Remove cells using recombinant trypsin, plant half the cells in a new T-75 flask
  - Repeat at day 14
- IFA detection day 21
  - Fix cells and stain cells on one set of slides with FITC-conjugated anti-PCV-2 antiserum, and another with anti-PCV-1/2 antiserum followed by FITC-conjugated anti-species secondary antibody.
  - Examine by epifluorescence microscopy

Quantitative infectivity in PK-15 with IF or CPE readout

- Plant PK-15 cells in 96-well plates
- Infect with dilution series of sample
- IFA - after 1 week, fix and stain for PCV-1 and PCV-2;
- CPE - review separate cultures for CPE after 10 days
- Calculate $TCID_{50}$ (SK)

Standards and Controls

- Infectivity
  - BioReliance PCV-1 and PCV-2
  - Merck PCV-1 and two PCV-2 isolates
- PCR
  - Plasmid encoding the PCV-2 rep gene
  - MRC-5 DNA, medium, buffers/diluents

Results

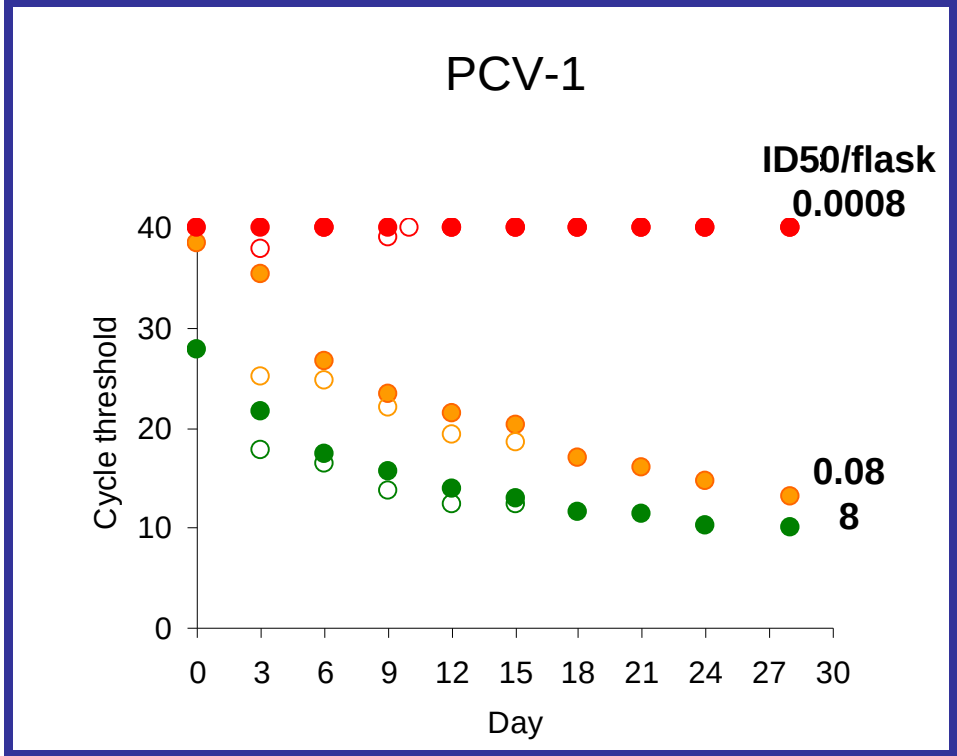
Characterization of PCV strains

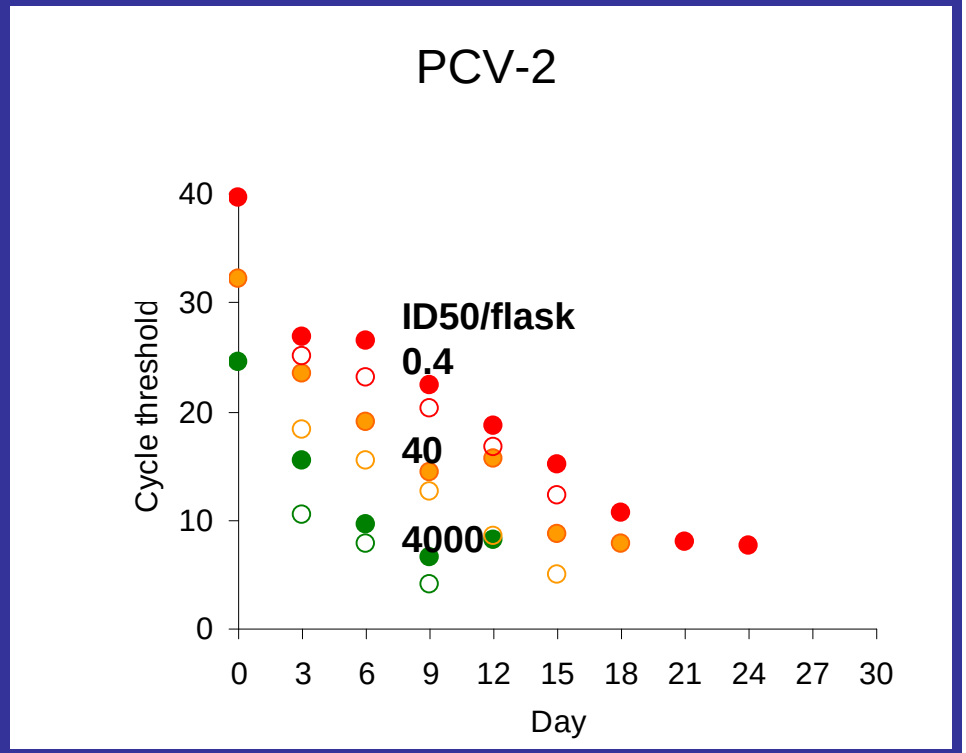
Virus	DNase-resistant genome copy titer (copies/mL)	IF-based infectious titer (ID50/mL)	CPE-based titers (ID50/mL)
PCV-1 BioReliance	1×10^{10}	8×10^{2}	Not cytopathic
PCV-2 BioReliance	8×10^{10}	2×10^{5}	1×10^{3}
PCV-1 Merck	5×10^{7}	Not determined	
PCV-2 isolate 1 Merck	2×10^{8}		
PCV-2 isolate 2 Merck	2×10^{7}		

PK-15 cells, 3-day splits, QPCR

PK-15 cells and supernatants are similarly sensitive

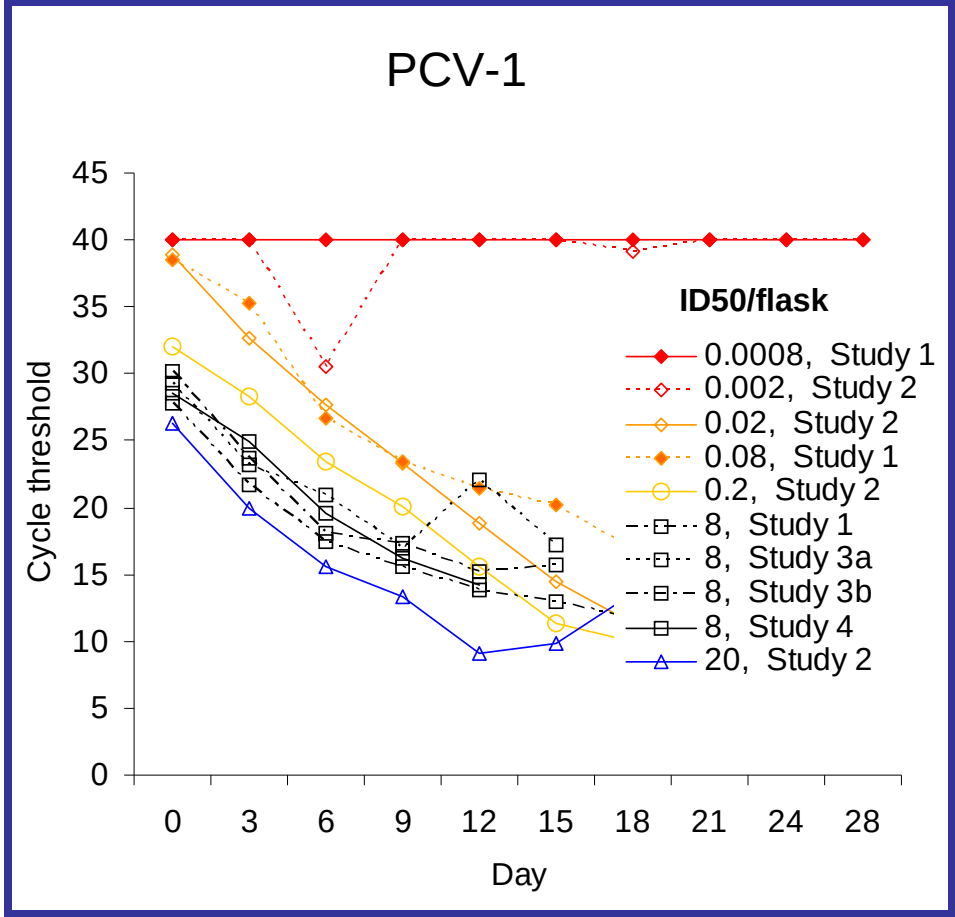
Culture supernatant, filled symbols
Cells, open symbols

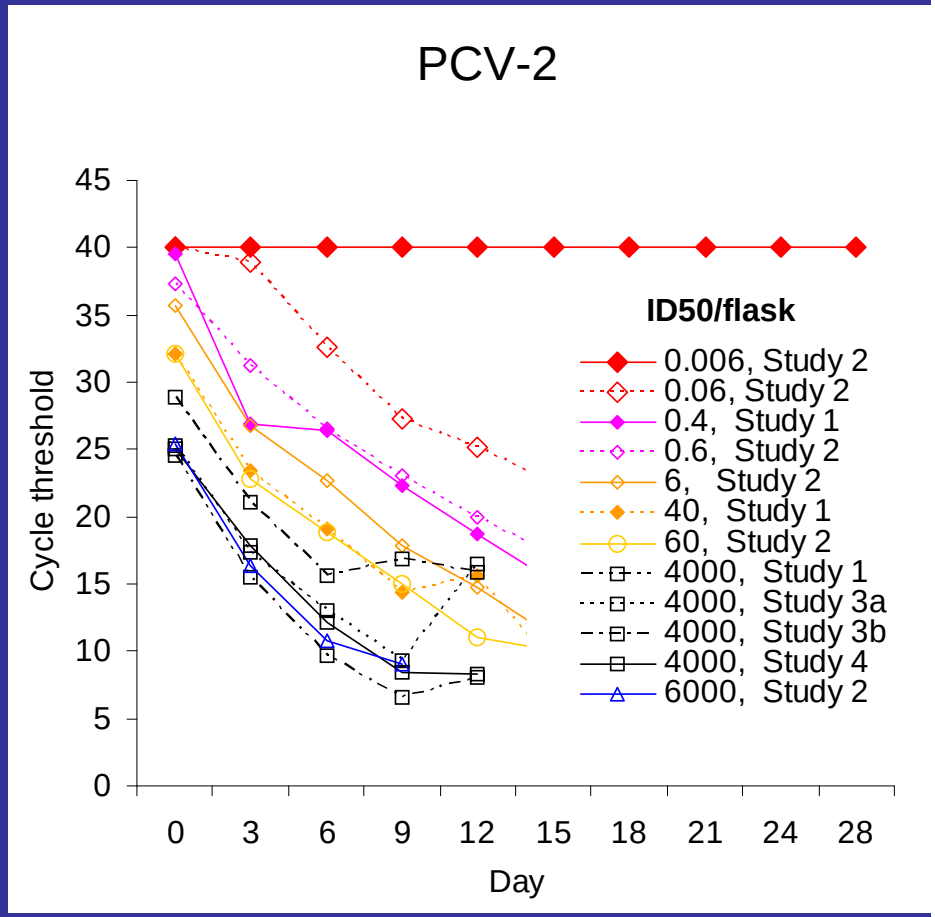




Dilutions of standards are relatively reproducible across runs

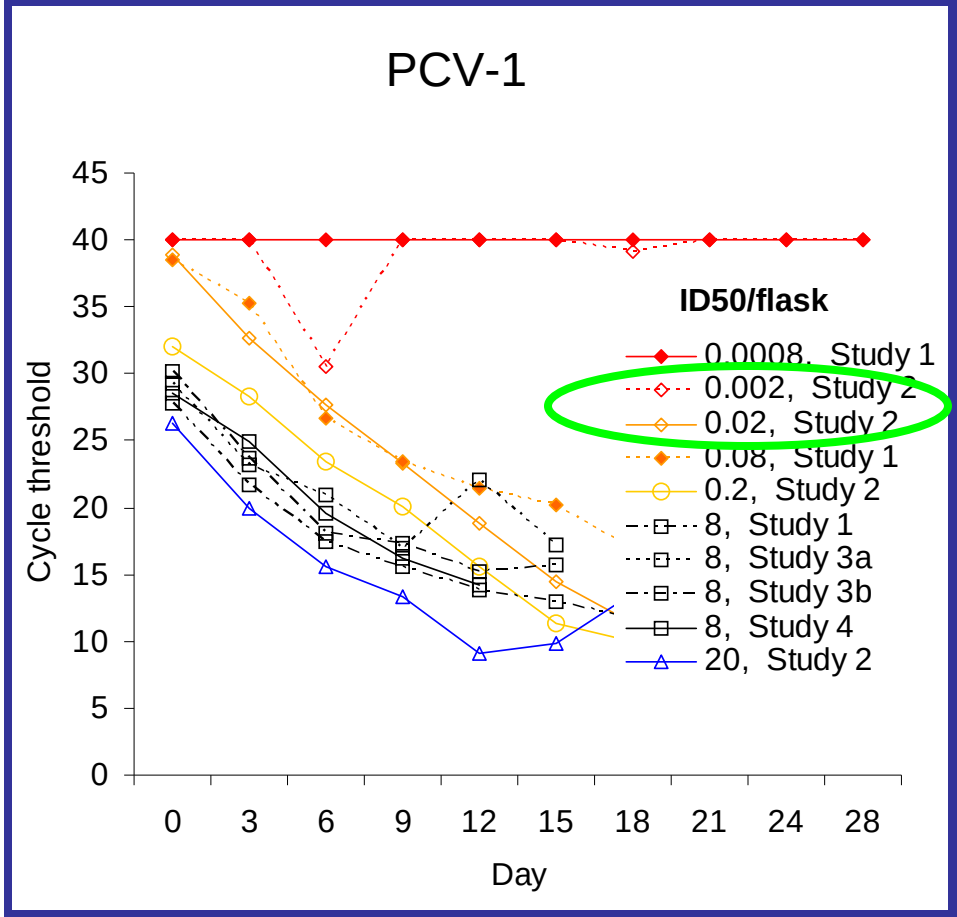
Culture supernatants tested

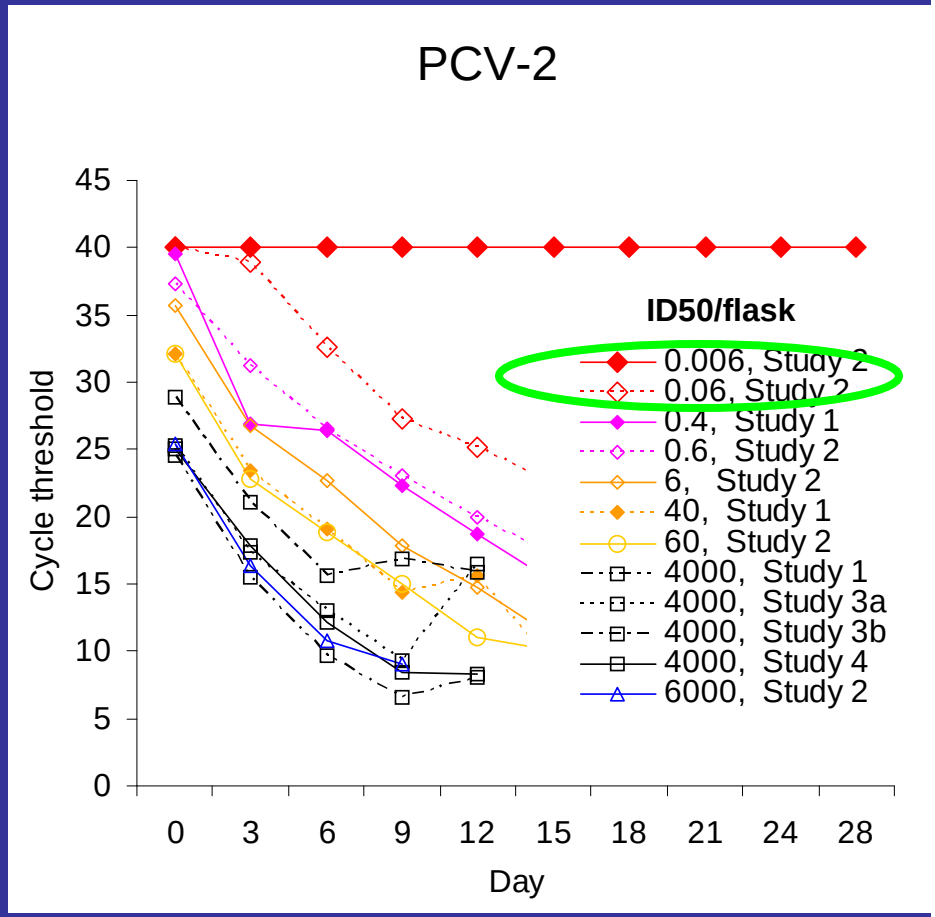




Appears that the 96-well IF titration assay under-estimated infectious PCV relative that detectable in flask assay

Culture supernatants tested





A comparative sensitivity study

What is the highest dilution (lowest amount) of 5 PCV strains that is detected in the respective methods?

If similar, then little justification for further development of alternative method.

Treatments

- PT-1 cells with 7-day splits and IF detection
  - (“9 CFR 113-like”)
- PK-15 cells with 3-day splits and DNA detection in culture supernatants (and observation for CPE)

All else equal…

- 5 PCV strains (two PCV-1, three PCV-2)
- 10-fold dilution series of each
- Same inoculation volume & adsorption conditions for each assay
- Incubations, splits, readouts as described previously

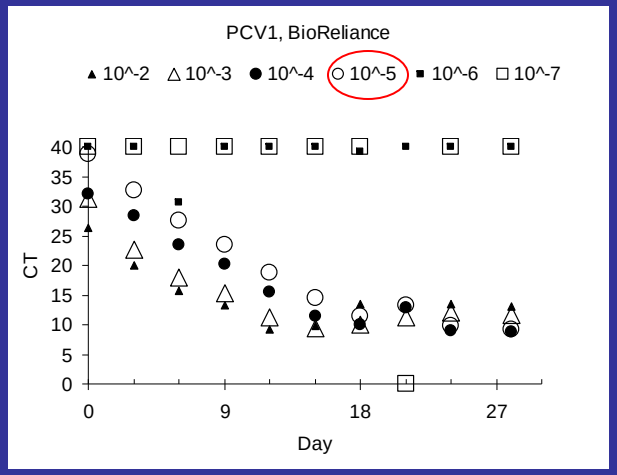
PT-1 cells, 7-day splits, IF readout

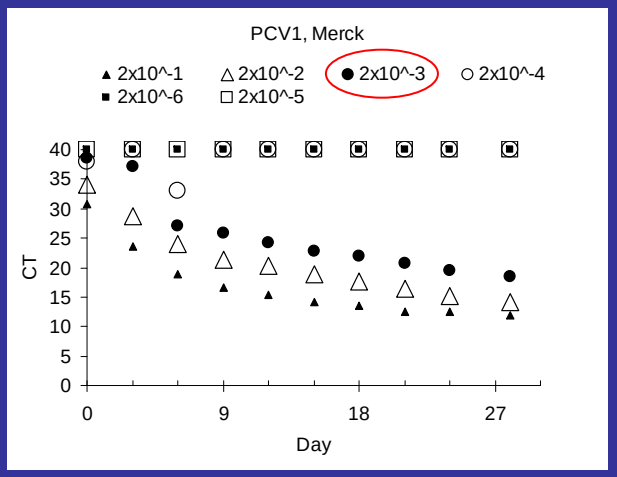
Virus strain Antisera	Dilutions							
PCV-1 Merck	**$2x10^{-1}$**	**$2x10^{-2}$**	**$2x10^{-3}$**	**$2x10^{-4}$**	**$2x10^{-5}$**	**$2x10^{-6}$**	**$2x10^{-7}$**	**$2x10^{-8}$**
Anti-PCV-1/2	-	-	-	-	-	-	-	-
Anti-PCV-2	-	-	-	-	-	-	-	-
PCV-2 isolate 1 Merck								
Anti-PCV-1/2	+	+	-	-	-	-	-	-
Anti-PCV-2	+	-	-	-	-	-	-	-
PCV-2 isolate 2 Merck								
Anti-PCV-1/2	-	-	-	-	-	-	-	-
Anti-PCV-2	-	-	-	-	-	-	-	-
	Dilutions							
PCV-1 BioReliance	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**10^{-8}**	**10^{-9}**
Anti-PCV-1/2	-	-	-	-	-	-	-	-
Anti-PCV-2	-	-	-	-	-	-	-	-
PCV-2 BioReliance								
Anti-PCV-1/2	+	+	+	-	-	-	-	-
Anti-PCV-2	+	+	+	-	-	-	-	-

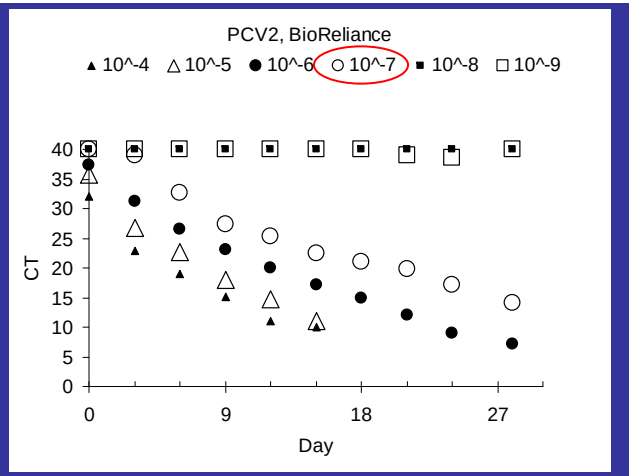
PK-15 cells, 3-day splits, CPE readout

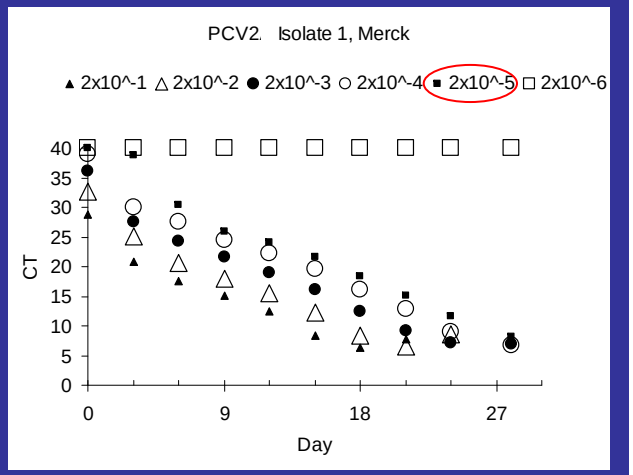
	Dilutions							
Merck strains	**$2x10^{-1}$**	**$2x10^{-2}$**	**$2x10^{-3}$**	**$2x10^{-4}$**	**$2x10^{-5}$**	**$2x10^{-6}$**	**$2x10^{-7}$**	**$2x10^{-8}$**
PCV-1	-	-	-	-	-	-	-	-
PCV-2 Isolate 1	100% CPE Day 21	100% CPE Day 24	50% CPE Day 28	-	-	-	-	-
PCV-2 Isolate 2	-	-	-	-	-	-	-	-
	Dilutions							
BioReliance strains	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**10^{-8}**	**10^{-9}**
PCV-1	-	-	-	-	-	-	-	-
PCV-2	80% CPE Day 9	50% CPE Day 9	80% CPE Day 15	40% CPE Day 15	80% CPE Day 28	-	-	-

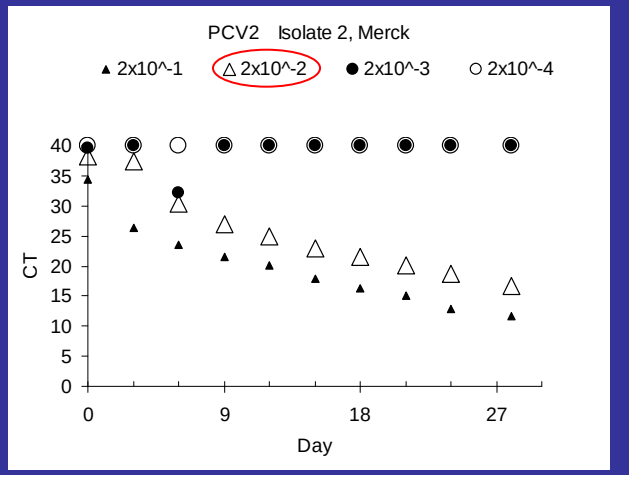
PK-15 cells, 3-day splits, QPCR readout











Comparative study overlay

	Highest dilution at which PCV was detected							
Merck Strains	**$2x10^{-1}$**	**$2x10^{-2}$**	**$2x10^{-3}$**	**$2x10^{-4}$**	**$2x10^{-5}$**	**$2x10^{-6}$**	**$2x10^{-7}$**	**$2x10^{-8}$**
PCV-1			PK-15 QPCR					
PCV-2 Isolate 1	PT-1 IFA		PK-15 CPE		PK-15 QPCR			
PCV-2 Isolate 2		PK-15 QPCR						
BioReliance strains	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**10^{-8}**	**10^{-9}**
PCV-1				PK-15 QPCR				
PCV-2			PT-1 IFA		PK-15 CPE	PK-15 QPCR		

Conclusions

- ***As performed***, the PT-1 cells with 7 day splits and IFA readout appeared less sensitive compared with the PK-15 QPCR method, and perhaps even unsuitable for PCV-1
- There does appear to be justification to explore an alternative approach to an *in vitro* PCV infectivity assay
- Specific treatment effects remain to be determined
- Differences between Merck PCV-2 isolates may be attributable in part to culture history

Next steps

- Could dissect main effects/interactions
  - Cells
  - Split schedule
  - IFA vs molecular readout
  - Infection approach
  - Proportion of cells maintained in culture
- Could explore quantitative format for PK-15 cells using 3-day split schedule and molecular readout

Acknowledgements

- Merck
  - Oversight committees

- BioReliance
  - Audrey Chang, Linda Roy, Anton Steuer, Linet Kiplagat, Jose Gutierrez, Chuck Pals, Phil Tedeschi, Sailaja Koduri, John Kolman