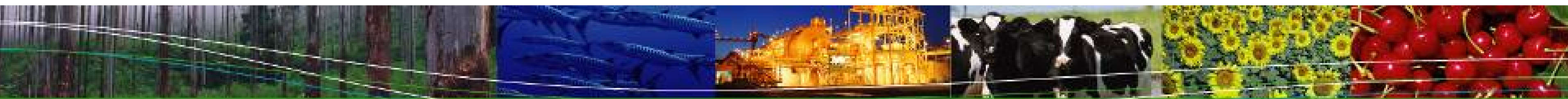
Responding to the challenges of emergency disease outbreaks – lessons from an animal health perspective

P.D. Kirkland.

Why do animal disease emergencies attract attention?

- Australia is free of a number of major diseases of animals (geographic isolation, eradication);
- Animal health status can have implications for public health, productivity and trade;
- New or emerging diseases of animals attract attention internationally - >70% of new human infectious diseases have an animal origin;
- Many drivers for control of emergency animal diseases;
- Often a goal to achieve regional or national freedom;
- Involves both agent detection and serology.



What can we learn ?

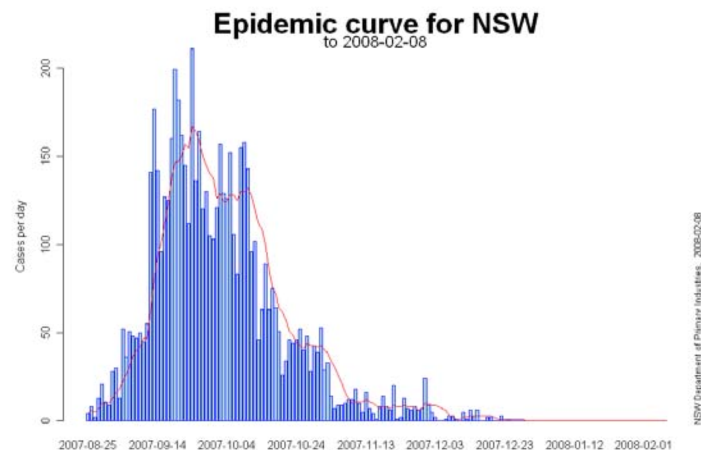
- Application of high through-put testing for an animal disease emergency – what do we do?
- Review of issues that impinge on high a through-put capability;
- Are the principles of a high through-put system relevant to ‘routine’ diagnostic applications?
- Quality management considerations & issues arising during routine diagnostic testing;
- Are ‘traditional’ diagnostic methods still relevant?

What methods are used?

- Agent detection - culture, antigen ELISA, PCR/qPCR, IFA all utilised during diagnosis and eradication;
- Development of “real time” PCR (qPCR) adds a new dimension to pathogen detection and makes large scale testing feasible;
- Serology (ELISA, HI, VNT) broadly applied to screen populations for presence of infection and to provide ‘proof of freedom;
- ELISAs or microsphere-based multiplex assays matched with a vaccine provide DIVA capability.

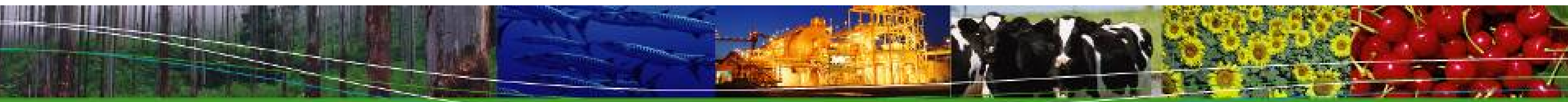
No limit to applications but need for continual refinements

- Responding to an exotic disease outbreak - Equine influenza (AI)
- Large scale monitoring of oysters for herpesvirus infection (OsHV-1);
- Discovery of a new pestivirus in pigs;
- BVDV testing on hair samples
- Meeting the demands of testing for a high consequence zoonotic pathogen - Hendra virus



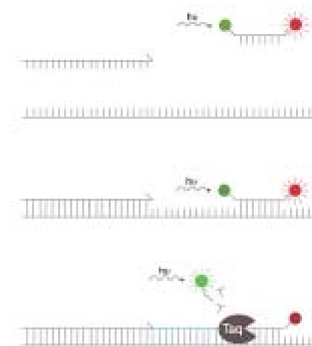
Gel based PCR - disadvantages

- High potential for cross contamination without segregation of work areas and very good laboratory practices
- Labor intensive;
- Limited daily capacity;
- Expensive when all costs calculated
- Can lack specificity



Real time PCR - advantages

- Marked reduction in likelihood of cross contamination;
- Reduced labor requirements;
- Significant cost reduction;
- Higher specificity with probe based assays;
- Short assay times and rapid turn around;
- Capacity for large scale testing;
- Quantification possible
- Greater potential for multiple pathogen detection (multiplexing)



Limitations to qPCR capacity

What did we learn from the equine influenza outbreak?

- Specimen receival and handling;
- Sample processing;
- Nucleic acid extraction and purification;
- High throughput qRT-PCR

Limitations to qPCR capacity

What did we learn from the equine influenza outbreak?

- Specimen reception and registration by far the greatest bottleneck;

  - Reduced by data entry at point of collection;

  - Assisted by bar coding of documents and collection materials.

Solutions: Specimen handling

THE GOAL: TO MINIMISE HANDLING and REDUCE STEPS AT ANY STAGE

Specimens:

- Require a consistent specimen type and packaging;
- Samples handled in 10 x 12 matrix from unpacking to disposal – avoid double handling;
- Find specimen type that requires minimal processing

Solutions: Specimen processing

THE GOAL: TO MINIMISE HANDLING and PROCESSING STEPS

Specimen processing:

- aim for liquid format (re-evaluate biology of disease)
- Avoid processing whenever possible (eg swabs in custom vs commercial transport medium);
- Body fluids – little or no processing;
- Serum vs whole blood;
- Tissues – swabs from cut surface;
  - enzymatic digestion;
- Semen, faeces – dilute if necessary (centrifuge only if essential);

Solutions: Equipment format

THE GOAL: TO MINIMISE HANDLING and REDUCE STEPS AT ANY STAGE

Equipment:

- Integration from 'start to finish':

  - compatible formats to minimise handling

  - 8 x 12 matrix the key

- From RNA extraction through to PCR & disposal;
- Appropriate degree of automation – fully automated may not be best

Nucleic acid extraction

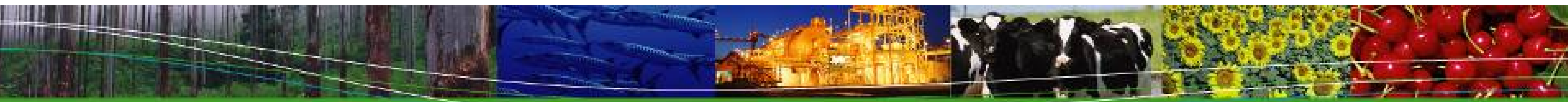
The limiting factor to widespread use and high throughput PCR

The aim: single system and chemistry for all samples and assays

- DNA and RNA extraction using a magnetic bead based kit (MagMax 96 viral RNA) combined with a magnetic particle handling system (KF 96) – numerous sample types on same plate (swabs, fluids, blood, tissue extracts, semen, faeces, cultures)

- Extraction of total nucleic acid – 92 samples plus controls – ~18 mins plus plating time

- Capacity for >6,000 extractions per day (2-3 technicians, 3 shifts, 2 machines)



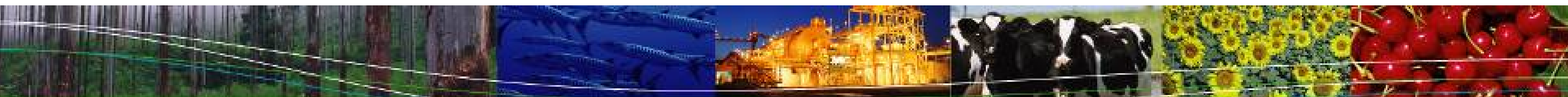
qPCR and qRT-PCR

The aim: single chemistry and format for all assays – DNA & RNA

- qRT-PCR master mix for all assays;
- Can be supplied in small or large kits;
- Fast RT (10 mins – significant reduction, robust with field material)
- Standard assay conditions for almost all assays;
- Premix in large batches (mastermix, primers, probe) – 'ready to use' (plus enzyme) – reduces time, aids standardisation;
- Assay set up and run time about 90 mins (<60 mins in Fast mode)
- Capacity for >3500 assays/day (incl extractions – 3 technicians, 3 shifts, 3 PCR cyclers)

Expectations of a high throughput system

- Maximise number of samples tested per day;
- Maintain rapid turn around;
- Accuracy of results not affected;
- Able to respond quickly to changing needs;
- Cost effective;
- Does not compromise biosecurity;
- Routine diagnostic work not compromised by a disease emergency



A high throughput system – some achievements

Equine Influenza outbreak:

- 21,000 accessions: a 10-15 fold increase in accessions and samples

  - qRT-PCR – 72,000 assays completed in 5 mths

  - >30,000 in 4 week period

  (3 technicians, 2 shifts: 2,300 in one day plus “business continuity” – total 3,000 PCRs)

- Maintained rapid turn around: ‘same day’ – ‘urgent’ samples 3 hrs;

Can systems that support high throughout be adapted for small scale testing?

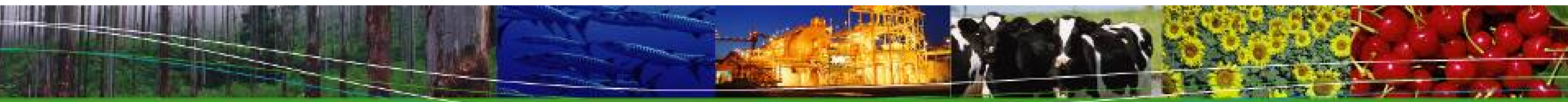
- Cost is a major consideration for small numbers of samples

  – what can be done to lower costs? (No Medicare for cows!!)

- Extractions – multiple sample types (anything!!) can be batch processed on same plate under same extraction conditions (limited prior processing);

- PCR – Different assays can be run on same plate at same time: Either as separate qPCR and qRT-PCR assays ("multi-tasking") or as multiplexed assays if same specimens are tested;

- Single chemistry for nucleic acid extractions and qPCR/qRT-PCRs minimise cost and maximise purchasing power for any lab needs;



Impact of sample collection methods and sample quality

- To what extent do sample collection methods and sample quality matter with highly sensitive assays?
- Does the environment in which samples have been collected make any difference?
- Do we need a full tube of unclotted blood when only 50uL is extracted?

Quality management considerations - sample collection

Factors for consideration:

- Is the weak positive result real?
- Is it a laboratory error or contamination
- Do we set a cut-off and avoid weak positives?

OR

Could these results be significant?

-Is the weak positive an indication of poor sample quality?

-Could it be the result of environmental contamination?

- Has there be any vaccine used or handled in the sample collection area?

Quality management considerations - sample type and quality

Factors for consideration:

- Samples:

  - type (eg serum vs plasma vs whole blood)

  - volume, additives & preservatives

  - quality/storage conditions

  - are old samples poor quality?

Which is the best sample?

Quality management considerations - sample volume & additives

Does sample volume & anticoagulant affect testing of whole blood?

Samples spiked with BHV-1 (same virus concentration per unit volume)

Volume (blood or PBS)	PBS only	PBS in EDTA	PBS in Heparin	Blood in EDTA	Blood in Heparin
0.5 mL	29.99				
1 mL	29.93				
2 mL	29.30				
4 mL	29.85				
6 mL	30.03				
8 mL	30.15				
10 mL	29.90				
Mean	**29.88**				

Tested neat, 25 ul sample

Quality management considerations - sample volume & additives

Does sample volume & anticoagulant affect testing of whole blood?

Samples spiked with BHV-1 (same virus concentration per unit volume)

Volume (blood or PBS)	PBS only	PBS in EDTA	PBS in Heparin	Blood in EDTA	Blood in Heparin
0.5 mL	29.99	29.86			
1 mL	29.93	30.23			
2 mL	29.30	30.15			
4 mL	29.85	30.09			
6 mL	30.03	29.89			
8 mL	30.15	30.25			
10 mL	29.90	29.90			
Mean	**29.88**	**30.05**			

Tested neat, 25 ul sample

Quality management considerations - sample volume & additives

Does sample volume & anticoagulant affect testing of whole blood?

Samples spiked with BHV-1 (same virus concentration per unit volume)

Volume (blood or PBS)	PBS only	PBS in EDTA	PBS in Heparin	Blood in EDTA	Blood in Heparin
0.5 mL	29.99	29.86	45.00		
1 mL	29.93	30.23	45.00		
2 mL	29.30	30.15	33.72		
4 mL	29.85	30.09	29.64		
6 mL	30.03	29.89	29.59		
8 mL	30.15	30.25	29.87		
10 mL	29.90	29.90	29.78		
Mean	**29.88**	**30.05**			

Tested neat, 25 ul sample

Quality management considerations - sample volume & additives

Does sample volume & anticoagulant affect testing of whole blood?

Samples spiked with BHV-1 (same virus concentration per unit volume)

Volume (blood or PBS)	PBS only	PBS in EDTA	PBS in Heparin	Blood in EDTA	Blood in Heparin
0.5 mL	29.99	29.86	45.00	30.37	
1 mL	29.93	30.23	45.00	30.89	
2 mL	29.30	30.15	33.72	31.42	
4 mL	29.85	30.09	29.64	30.61	
6 mL	30.03	29.89	29.59	31.55	
8 mL	30.15	30.25	29.87	30.84	
10 mL	29.90	29.90	29.78	31.47	
Mean	**29.88**	**30.05**		**31.02**	

Tested neat, 25 ul sample

Quality management considerations - sample volume & additives

Does sample volume & anticoagulant affect testing of whole blood?

Samples spiked with BHV-1 (same virus concentration per unit volume)

Volume (blood or PBS)	PBS only	PBS in EDTA	PBS in Heparin	Blood in EDTA	Blood in Heparin
0.5 mL	29.99	29.86	45.00	30.37	42.16
1 mL	29.93	30.23	45.00	30.89	32.21
2 mL	29.30	30.15	33.72	31.42	30.61
4 mL	29.85	30.09	29.64	30.61	31.30
6 mL	30.03	29.89	29.59	31.55	30.76
8 mL	30.15	30.25	29.87	30.84	30.44
10 mL	29.90	29.90	29.78	31.47	31.21
Mean	**29.88**	**30.05**		**31.02**	

Tested neat, 25 ul sample

Quality management considerations - sample volume & additives

Does sample volume & anticoagulant affect testing of whole blood?

Samples spiked with BVDV (same virus concentration per unit volume)

Volume (blood or PBS)	PBS only	PBS in EDTA	PBS in Heparin	Blood in EDTA	Blood in Heparin
0.5 mL	26.17				
1 mL	26.39				
2 mL	26.66				
4 mL	26.52				
6 mL	26.68				
8 mL	26.50				
10 ml	26.50				
Mean	**26.49**				

Tested neat, 25 ul sample

Quality management considerations - sample volume & additives

Does sample volume & anticoagulant affect testing of whole blood?

Samples spiked with BVDV (same virus concentration per unit volume)

Volume (blood or PBS)	PBS only	PBS in EDTA	PBS in Heparin	Blood in EDTA	Blood in Heparin
0.5 mL	26.17	26.35			
1 mL	26.39	26.39			
2 mL	26.66	26.18			
4 mL	26.52	26.32			
6 mL	26.68	26.18			
8 mL	26.50	26.18			
10 ml	26.50	26.15			
Mean	**26.49**	**26.25**			

Tested neat, 25 ul sample

Quality management considerations - sample volume & additives

Does sample volume & anticoagulant affect testing of whole blood?

Samples spiked with BVDV (same virus concentration per unit volume)

Volume (blood or PBS)	PBS only	PBS in EDTA	PBS in Heparin	Blood in EDTA	Blood in Heparin
0.5 mL	26.17	26.35	45.00		
1 mL	26.39	26.39	45.00		
2 mL	26.66	26.18	45.00		
4 mL	26.52	26.32	45.00		
6 mL	26.68	26.18	38.20		
8 mL	26.50	26.18	35.10		
10 ml	26.50	26.15	33.57		
Mean	**26.49**	**26.25**			

Tested neat, 25 ul sample

Quality management considerations - sample volume & additives

Does sample volume & anticoagulant affect testing of whole blood?

Samples spiked with BVDV (same virus concentration per unit volume)

Volume (blood or PBS)	PBS only	PBS in EDTA	PBS in Heparin	Blood in EDTA	Blood in Heparin
0.5 mL	26.17	26.35	45.00	45.00	
1 mL	26.39	26.39	45.00	33.78	
2 mL	26.66	26.18	45.00	33.07	
4 mL	26.52	26.32	45.00	31.37	
6 mL	26.68	26.18	38.20	30.36	
8 mL	26.50	26.18	35.10	29.97	
10 ml	26.50	26.15	33.57	27.63	
Mean	**26.49**	**26.25**		**33.03**	

Tested neat, 25 ul sample

Quality management considerations - sample volume & additives

Does sample volume & anticoagulant affect testing of whole blood?

Samples spiked with BVDV (same virus concentration per unit volume)

Volume (blood or PBS)	PBS only	PBS in EDTA	PBS in Heparin	Blood in EDTA	Blood in Heparin
0.5 mL	26.17	26.35	45.00	45.00	45.00
1 mL	26.39	26.39	45.00	33.78	45.00
2 mL	26.66	26.18	45.00	33.07	45.00
4 mL	26.52	26.32	45.00	31.37	36.02
6 mL	26.68	26.18	38.20	30.36	35.50
8 mL	26.50	26.18	35.10	29.97	32.33
10 ml	26.50	26.15	33.57	27.63	32.17
Mean	**26.49**	**26.25**		**33.03**	

Tested neat, 25 ul sample

Quality management considerations – sample collection

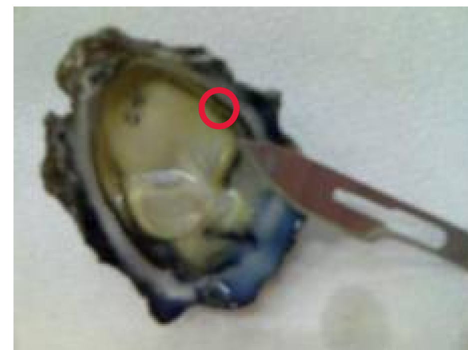
Factors for consideration:

- Sample collection

  - Are samples representative

  - Could there be cross contamination; environmental contamination - vaccine?

- Processing – how long are extracts stable; likely to be more non-specific amplification?
- Pooling – will it affect sensitivity?
- Dilution – will it reduce inhibitors/affect sensitivity?



Quality management considerations – extraction methods

- Nucleic acid extraction:

  - Method – manual vs automated methods, sample volume

- Some examples:

Quality management considerations - extraction methods

- Changes in wash times alone make a significant difference
- Reducing sample volume can make a difference

Laboratory	Lab 1		Lab 2		EMAI NSW	
Extraction	RNeasyPlus		Magmax Viral 96		Magmax Viral 96	
Sample volume in Extraction (ul)	100	100	50	50	50	50
Dilution of Blood in prior to Extraction	NIL	1:10	NIL	1:10	NIL	1:10
Elution volume (ul)	50	50	50	50	50	50
Dilution of RNA to assay	NIL	NIL	NIL	NIL	NIL	NIL
Threshold value	0.05	0.05	0.05	0.05	0.05	0.05
Master Mix	Ag Path-ID		Ag Path-ID		Ag Path-ID	
RNA volume to assay	2	2	5	5	5	5
Sample Id	Ct	Ct	Ct	Ct	Ct	Ct
BLU 1	NEG	36.8	25.04	25.34	Neg	23.57
BLU 3	36.85	NEG	21.26	23.55	Neg	25.02
BLU 9	NEG	NEG	24.17	25.72	Neg	28.25
BLU 15	NEG	35.9	22.98	25.13	22.29	26.16
BLU 16	NEG	NEG	25.97	26.83	28.15	29.79
BLU 23	NEG	33.2	21.37	22.84	Neg	24.05

Quality management considerations - extraction methods

- Changes in wash times alone make a significant difference
- Reducing sample volume can make a difference

Laboratory	Lab 1		Lab 2		EMAI NSW		EMAI Modified	
Extraction	RNeasyPlus		Magmax Viral 96		Magmax Viral 96		Magmax Viral 96	
Sample volume in Extraction (ul)	100	100	50	50	50	50	25	50
Dilution of Blood in prior to Extraction	NIL	1:10	NIL	1:10	NIL	1:10	NIL	Nil
Elution volume (ul)	50	50	50	50	50	50	50	50
Dilution of RNA to assay	NIL	NIL	NIL	NIL	NIL	NIL	NIL	NIL
Threshold value	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Master Mix	Ag Path-ID		Ag Path-ID		Ag Path-ID		Ag Path-ID	
RNA volume to assay	2	2	5	5	5	5	5	5
Sample Id	Ct	Ct	Ct	Ct	Ct	Ct	Ct	Ct
BLU 1	NEG	36.8	25.04	25.34	Neg	23.57	23.00	26.29
BLU 3	36.85	NEG	21.26	23.55	Neg	25.02	21.25	23.24
BLU 9	NEG	NEG	24.17	25.72	Neg	28.25	24.23	28.48
BLU 15	NEG	35.9	22.98	25.13	22.29	26.16	21.70	24.01
BLU 16	NEG	NEG	25.97	26.83	28.15	29.79	25.53	27.27
BLU 23	NEG	33.2	21.37	22.84	Neg	24.05	21.34	28.71

Quality management considerations - extraction methods

Factors for consideration:

- Nucleic acid extraction:

  - Use of internal controls – are they worth the effort?

  - What options are there and what needs to be done for their routine use?

- Some examples:

Quality management considerations - use of internal controls

BTV Positive Blood - Neat		
BTV Sample	BTV qPCR (ct)	IC qPCR (ct)
1	-	-
2	-	-
3	-	-
4	-	-
5	30	34
6	-	-
positive control	29	29
Negative control	-	30
Blank	-	-

IC Ct range in BTV assay 29-31

Quality management considerations - use of internal controls

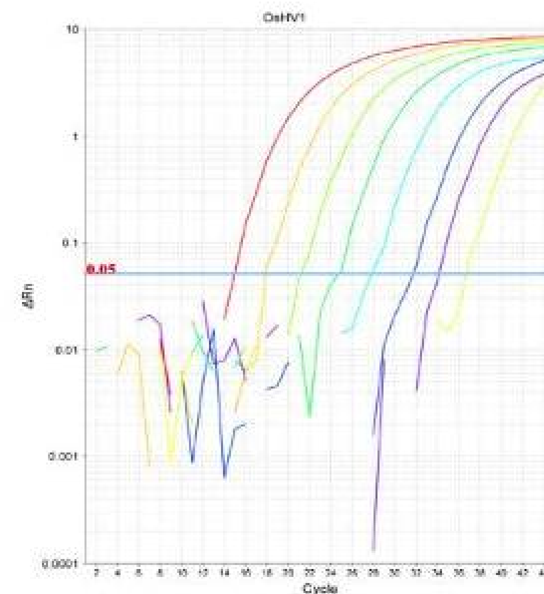
BTV Positive Blood - Neat		
BTV Sample	BTV qPCR (ct)	IC qPCR (ct)
1	-	-
2	-	-
3	-	-
4	-	-
5	30	34
6	-	-
positive control	29	29
Negative control	-	30
Blank	-	-

BTV Positive Blood - Diluted 1/10		
BTV Sample	BTV qPCR (ct)	IC qPCR (ct)
1	27	34
2	25	32
3	28	31
4	25	31
5	29	30
6	24	30
positive control	29	29
Negative control	-	30
Blank	-	-

IC Ct range in BTV assay 29-31

Quality management considerations - reagents and controls

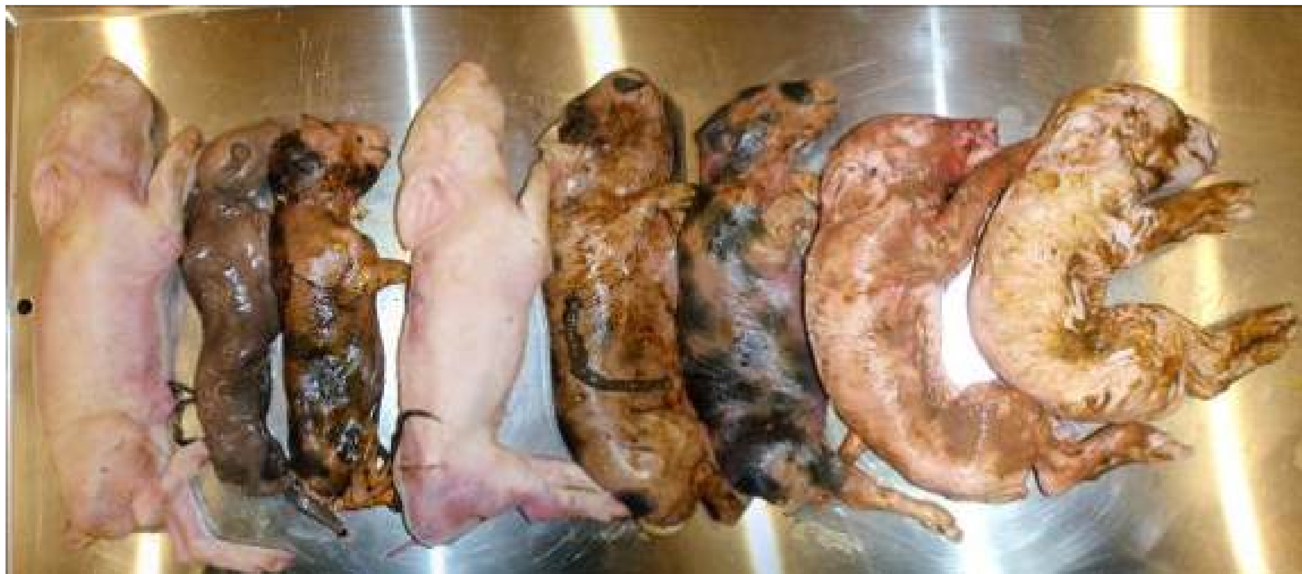
- Reagents & controls:
  - Mastermix & preparation – lot size, documentation of batches
  - Primers & probes (source, storage, documentation, handling)
  - Controls – positive, neg, blank – how many, single use aliquots, target range – what is acceptable?



Porcine myocarditis syndrome – a new virus

An exquisite example of the need to retain a wide range of skills through the application of and dependence on:

- Basic immunological and serological assays (initially AGID)
- Latest molecular tools – SISPA and nucleic acid sequencing
- A capacity to conduct experimental transmission and pathogenesis studies



Porcine myocarditis syndrome – pathology

- Still births and sudden death in neonatal pigs
- 2 affected farms – ~ 50,000 deaths
- Gross findings:

  - subcutaneous oedema;

  - cardiac enlargement;

  - increased pericardial, thoracic and abdominal fluid;

  - hepatic congestion

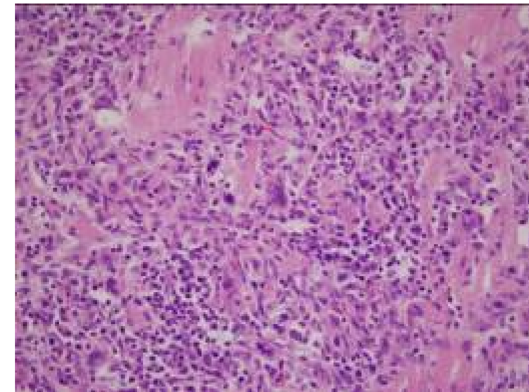
PMC Syndrome - pathology

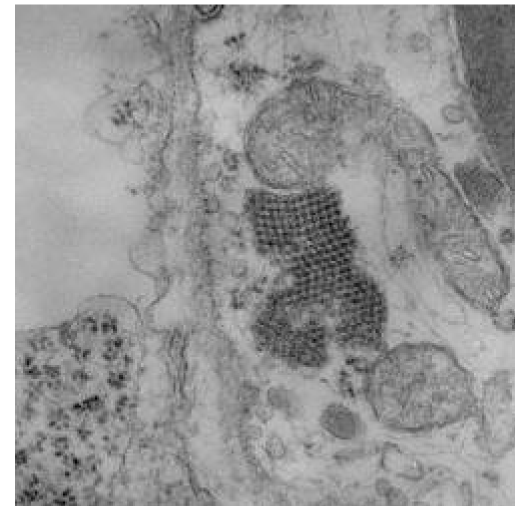
- Histopathology: acute to subacute multifocal, nonsuppurative myocarditis with myonecrosis;

  Sometimes non-suppurative pneumonitis, hepatitis, encephalitis and lymphadenitis

- Electron microscopy: crystalline arrays in vascular endothelial cells of heart. Approx 25 nm.

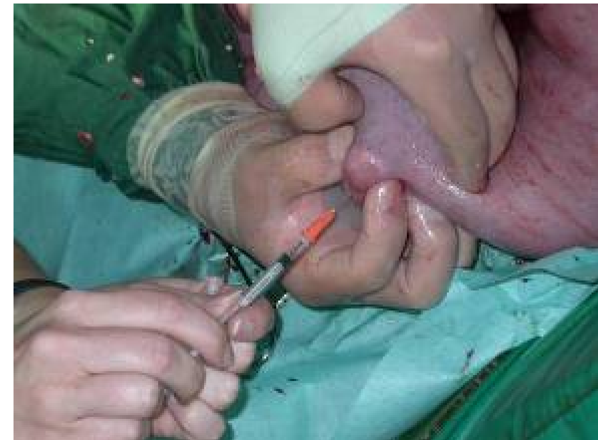
- Approx. 50% stillborns had increased IgG level – virus suspected.

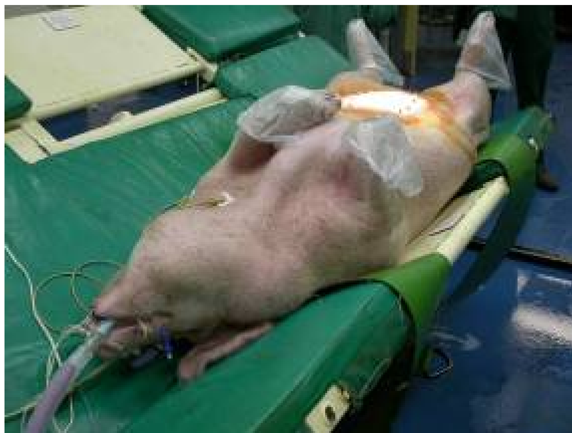


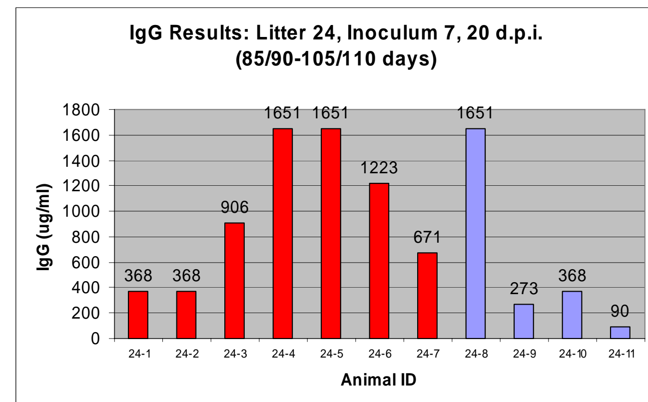


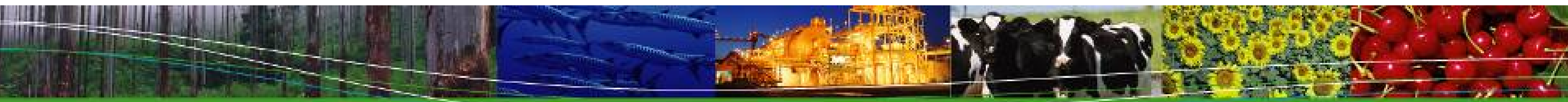
PMC investigations

- Foetal inoculation studies undertaken – evidence of *in utero* infection and lateral transmission of an infectious agent.



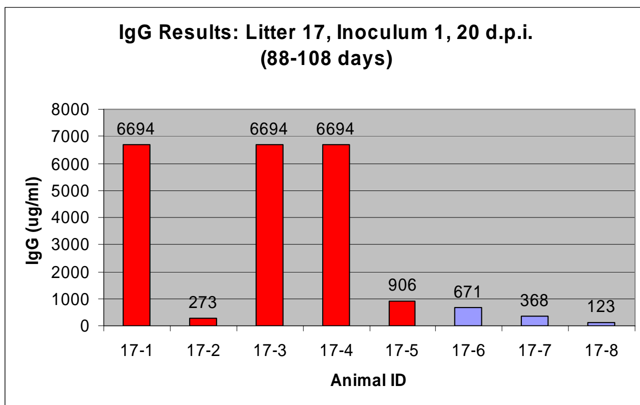


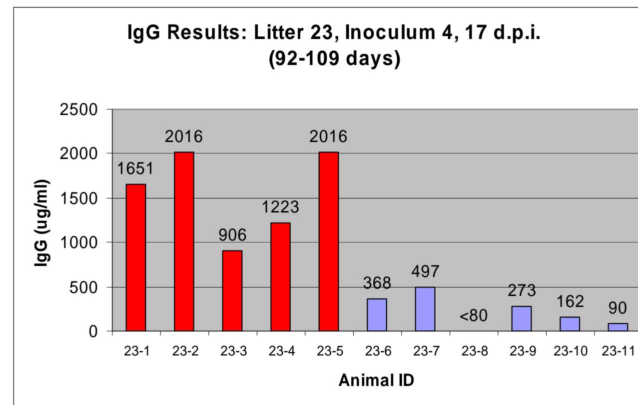


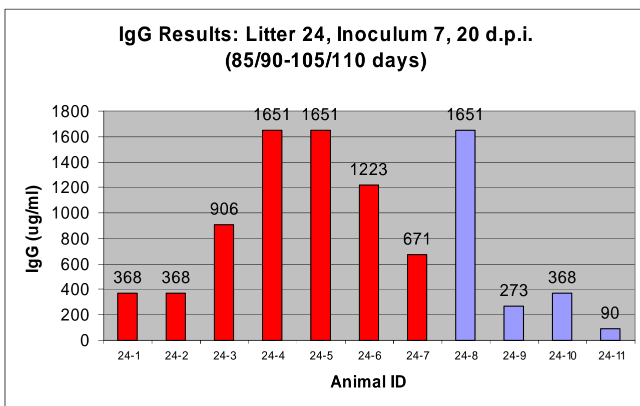


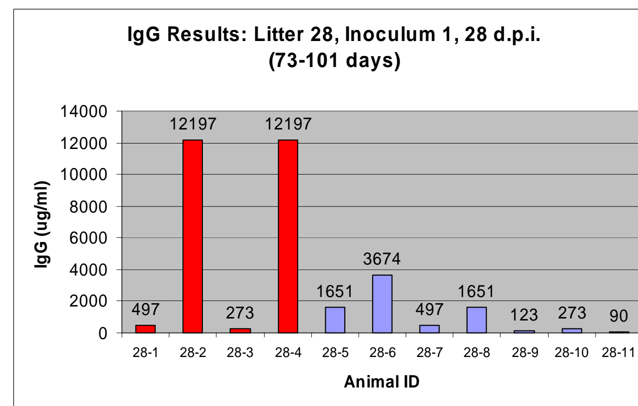
PMC investigations

Elevated foetal IgG levels indicate infectious agent and lateral transmission



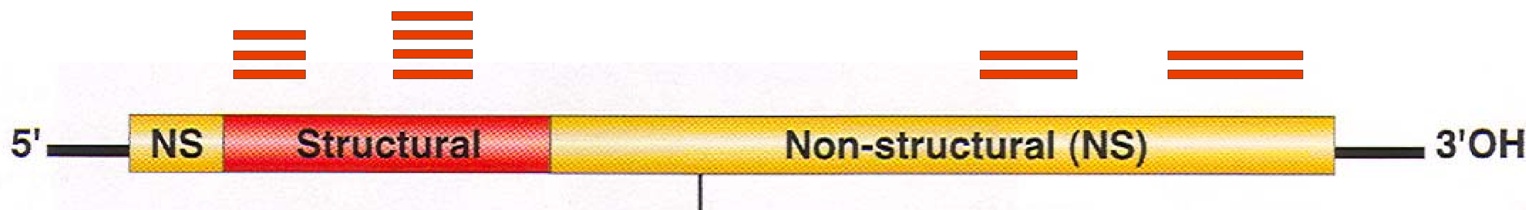






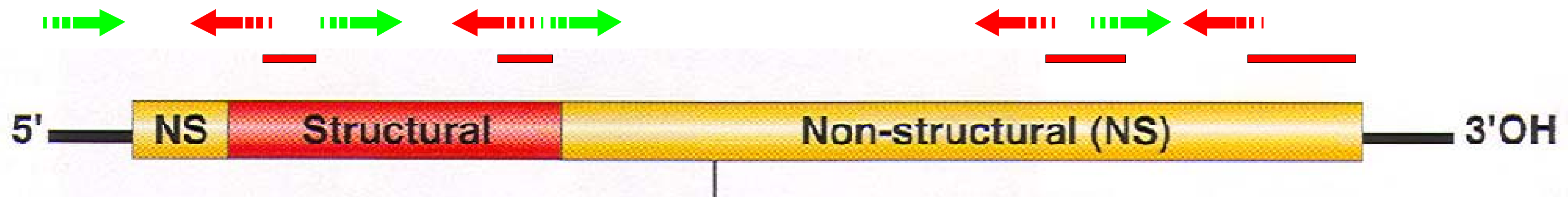
PMC investigations – agent identification

- Nucleic acid extracted from pooled serum from foetuses from 1 litter, 13 days post inoculation – all foetuses with crystalline arrays in myocardium
- DNA and RNA preparations completed for SISPA
- 350 DNA clones & 320 RNA clones submitted for nucleic acid sequencing
- No viral sequences identified in DNA clones
- 11 RNA clones contain sequence with homology to a pestivirus, from 4 regions of genome - 4 to the E^{rns} region, 3 to the p7 region, and 2 each to the NS5A and NS5B regions).



PMC investigations – molecular studies

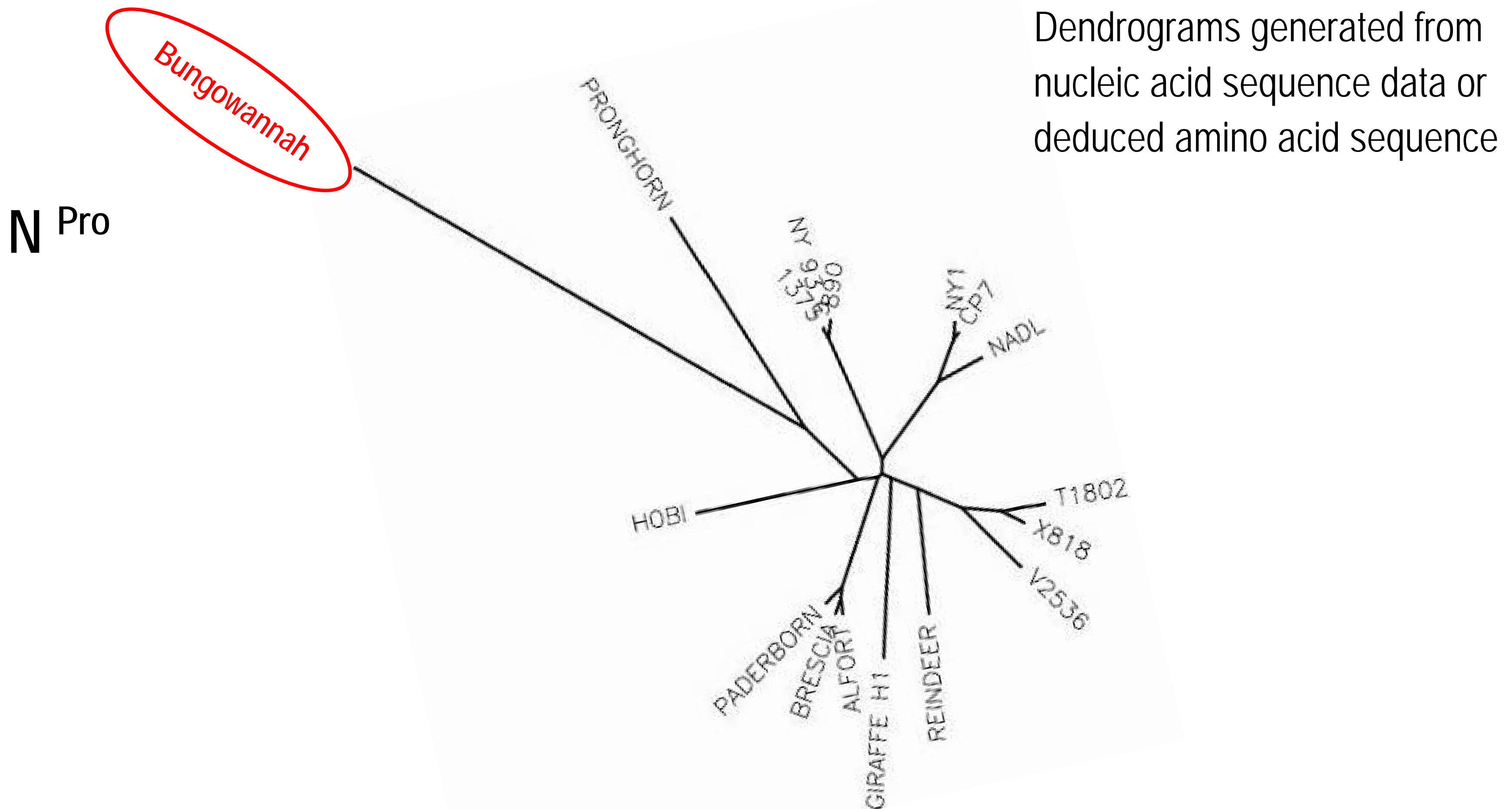
- Sequence extended by designing sets of overlapping primers, amplification by PCR then cloning and sequencing;
- Sequence consistent with the 5'UTR, N^{Pro} and E2 regions of a pestivirus genome was initially identified;
- Primer 'walking' & RACE used to obtain additional sequence;
- Some segments with homology only at amino acid level;
- Entire genome sequenced – 12.659Kb.

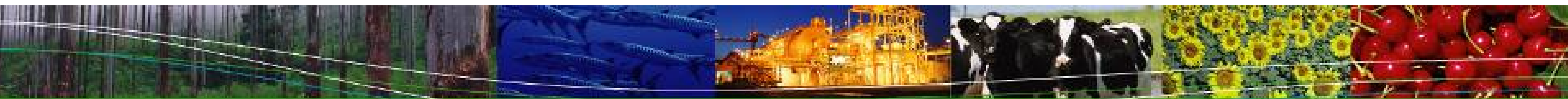


Bungowannah virus – phylogenetic studies

Dendrograms generated from nucleic acid sequence data or deduced amino acid sequence

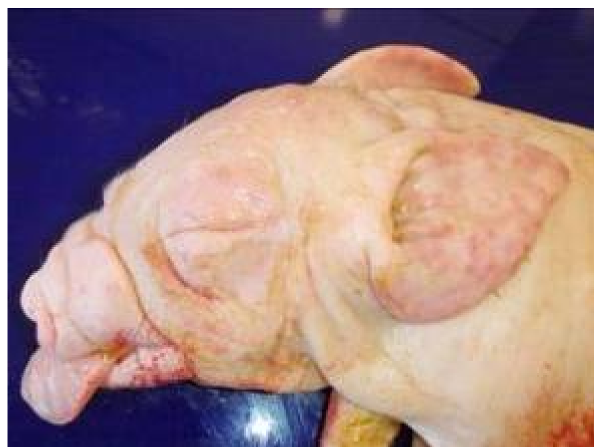
N^{Pro}

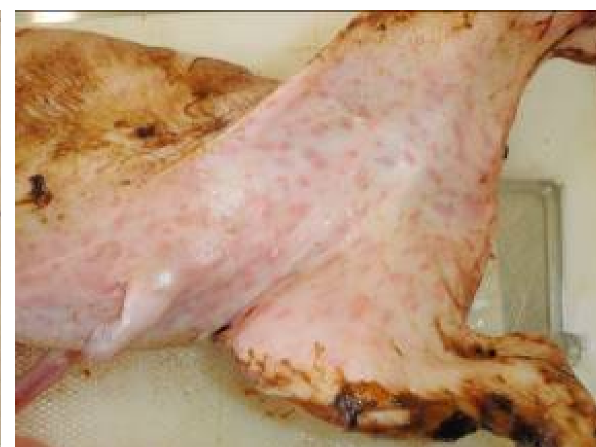


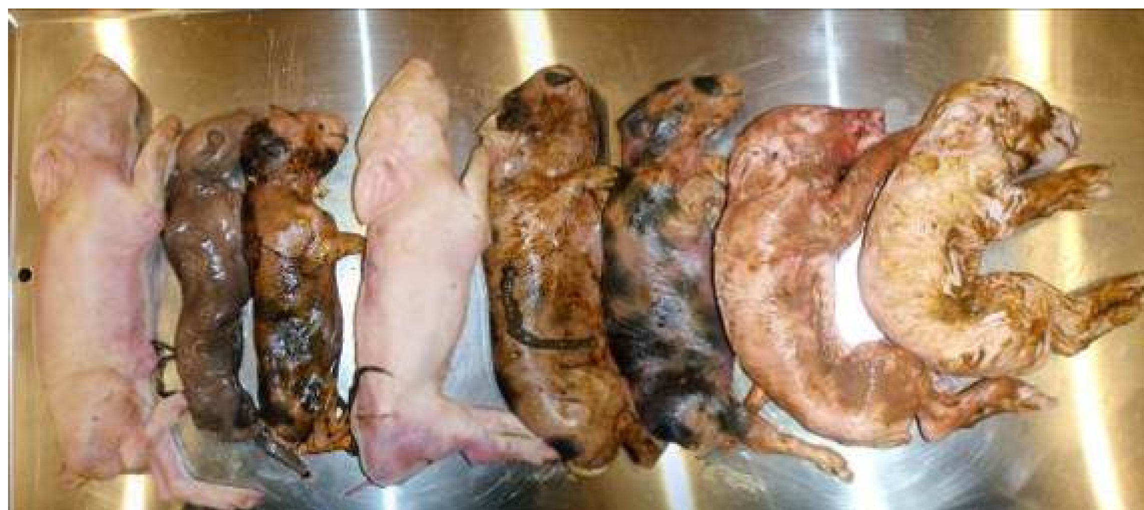


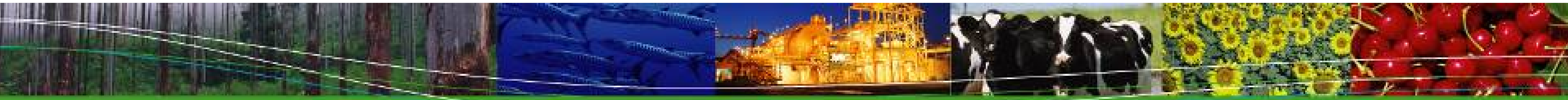
Bungowannah transmission studies in sows

Infection at D35









Caution!!

Real time PCR, high throughput sequencing and microarray technologies have the potential to revolutionise research and diagnosis in microbiobiology and virology

but

DO NOT ABANDON CLASSICAL TECHNIQUES

The key leads for the discovery of Bungowannah virus were a combination of AGID and ‘state of the art’ molecular techniques

Keys to a successful laboratory system

- Appropriately trained, skilled and committed staff

Thank you for your attention