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AUSTRALIAN Pork

Evaluation of diagnostic assays for porcine epidemic diarrhoea coronavirus

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Executive Summary

First recognised in England in the 1970's, Porcine Epidemic Diarrhoea Virus (PEDV) has been described as a re-emerging epizootic swine virus. Since the 1980's this virus has plagued the Asian pork industry, causing high mortality in neonatal piglets and has since demonstrated a somewhat endemic pattern. However, recent devastating outbreaks in the United States and subsequently Canada and Mexico have raised international awareness of this virus. In addition, several large-scale epidemics in South Korea, Japan and Taiwan have also attracted attention.

Another coronavirus, Porcine Delta Coronavirus (PDCoV) has also been detected both independently and concurrently with PEDV in faecal samples from clinical cases in the USA. The clinical impact is less severe than PEDV but PDCoV can still cause severe disease often indistinguishable from both PEDV and a third porcine coronavirus, Transmissible Gastroenteritis Virus (TGEV).

There is an apparent absence of disease resembling PEDV and PDCoV infections in Australia. However, there is no laboratory evidence to support this assumption. When PEDV was first discovered in the outbreak in the USA, diagnosis was complicated by cross reactivity from PDCoV in assays for the detection of PEDV. There has been no large-scale use of diagnostic tests for PEDV in Australia. Consequently, there is no appreciation of whether there may be other endemic, non-pathogenic coronaviruses circulating in the Australian pig herd and that may cause false positive results in PEDV assays. The aim of this project was to undertake an evaluation of diagnostic assays available internationally and to confirm that these have a high specificity (lack of false positive results) when testing samples from Australian pigs. This knowledge is invaluable in preparing for a response should there be an incursion of PEDV into Australia

Ensuring a PEDV-free status in Australia requires the capability to rapidly diagnose and exclude cases through the establishment of appropriately validated assays across the Laboratories for Emergency Animal Disease Diagnosis and Response (LEADDR) network. This study has evaluated four commercially available qRT-PCR assays and two 'in-house' assays provided by US diagnostic laboratories by the testing of a reference collection (n=141) and samples from Australian origin (n=484).

Through the completion of a national survey, this study has provided evidence for the freedom from PEDV and PDCoV in Australian pig populations. A total of 484 samples including rectal swabs (n=385) and environmental swabs (n=99) from 28 herds across the Australian pig producing population demonstrated negative results in six PEDV qRT-PCR assays and five PDCoV qRT-PCR assays.

This study has identified several viable options involving the use of commercially available assays, but consideration should be given to the PEDV and PDCoV qRT-PCR capabilities of the 'in-house' assays as these are considerably less expensive and present an alternative where delays in international transport may prevent the rapid delivery of commercial assays.

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1. Background to Research

Porcine epidemic diarrhoea virus (PEDV) is a large enveloped single-stranded RNA virus and member of the genera *Alphacoronavirus* in the family *Coronaviridae* (Jung and Saif, 2015, Lee, 2015). PEDV was first identified in England in the 1970's and has since been responsible for significant economic losses across both European and Asian swine industries, however recent outbreaks across the United States have crippled the American swine industry (Lee, 2015). With rapid spread across continental USA, the 2013-2014 outbreak led to annual losses to American pig producers of $900 million to $1.8 billion (Langel et al., 2016) . Nucleic acid sequencing and analysis of US virulent PEDV strains reveal a similar nucleotide homology (>99%) to strains from China, however the origin of PEDV in the US remains unknown (Stevenson et al., 2013). In December 2013 a variant strain of PEDV initially called "US variant" (later renamed S-INDEL) emerged and caused significantly less mortality than the previous virulent strain, but provides evidence of at least two genotypes of PEDV circulating in the USA (Wang et al., 2014, Jarvis et al., 2016). The variant strain of PEDV demonstrates a large deletion in the spike gene (S), a major structural protein of the virus (Masuda et al., 2015, Jarvis et al., 2016).

In 2014 in regions of the USA where PEDV is now endemic, a genetically distinct coronavirus belonging to the *Deltacoronavirus* genus, porcine delta coronavirus (PDCoV), was detected in faecal samples from clinical cases of enteritis (Homwong et al., 2016). Subsequent retrospective testing has confirmed that PDCoV was present in the USA as early as August 2013 (Sinha et al., 2015). Detection has occurred both independently and simultaneously with PEDV in some cases (Jung et al., 2015b, United States Department of Agriculture, 2016). In a large scale survey in the USA between July 2014 and March 2016, 12% of samples were positive for PEDV, 2.2% positive for PDCoV, and 1.8% were positive for both (United States Department of Agriculture, 2016). The pathogenicity of PDCoV has been confirmed experimentally and while the clinical severity is reported to be less severe than PEDV, PDCoV can cause severe atrophic enteritis accompanied by diarrhoea and/or vomiting (Jung et al., 2015b, Chen et al., 2015). The USA PDCoV sequences also share a high nucleotide identity with both Hong Kong (>98%) and Korean strains (>99%) (Lee and Lee, 2014, Wang et al., 2016).

Following faecal-oral transmission, infection with PEDV initially presents as a malabsorptive watery diarrhoea, vomiting, anorexia, and concurrent high mortality rates in pigs of all ages. The virus is known to exhibit more severe clinical signs in suckling pigs with 100% morbidity and 50-100% mortality (Stevenson et al., 2013). As pigs become older relatively milder clinical signs are observed and the mortality rate becomes progressively lower. The mechanisms by which PEDV affects suckling piglets versus weaned pigs have not yet been clearly identified, however factors such as slower turnover of enterocytes in suckling piglets has been hypothesised (Jung et al., 2015a). Surviving pigs show poor growth rates due to reduced feed intake and residual damage to the gastrointestinal tract (Curry et al., 2016). Clinical signs and histopathologic lesions are indistinguishable from other known pathogenic coronaviruses that primarily cause enteric disease including transmissible gastroenteritis virus (TGEV) in the Alphacoronavirus genus and PDCoV(Diel et al., 2016).

The similar clinical presentation of infection with the known coronaviruses highlights the critical importance of specific laboratory assays to differentiate between PEDV, PDCoV, and TGEV. Molecular diagnostic assays, especially qRT-PCR, have become the method of choice for the diagnosis of PEDV due to their reported sensitivity and specificity and rapid turnaround of results (Kim et al., 2000, Kim et al., 2001, Kim et al., 2007). A number of single and multiplex qRT-PCR assays have been developed for the diagnosis of PEDV and PDCoV (Wang et al., 2014, Kim et al., 2000, Kim et al., 2001, Kim et al., 2007). Duplex qRT-PCR assays have also been developed to differentiate between the two

circulating PEDV genotypes in the USA (Wang et al., 2014). Other diagnostic assays including virus isolation, immunofluorescence and immunohistochemistry are available for the detection of virus or viral proteins, however they often require clinical samples from the intestines of dead pigs and are relatively cumbersome to use resulting in longer viral detection times (Diel et al., 2016, Kim et al., 2007). qRT-PCR assays also have the advantage of providing a semi-quantitative result and can detect nucleic acid from inactivated, non-infectious samples.

Based on the apparent absence of disease resembling PEDV in the Australian porcine population, it is expected that this virus would have a devastating impact on production if introduced. In order to minimise the impact, early and rapid diagnosis, combined with stringent movement controls and biosecurity measures, is essential. The local evaluation of assays, by testing of samples from Australian pigs, in advance of any potential need is an important element of being prepared. Currently available PCR assays have been used to detect virus or viral proteins in a range of clinical and environmental samples including individual rectal swabs, intestinal and faecal samples, oral fluids and feed samples (Diel et al., 2016, Jung et al., 2015a, Bjustrom-Kraft et al., 2016). Development of a PCR assay to detect and differentiate PEDV from other known pathogenic coronaviruses requires the ability to target a specific region of the viral genome of known circulating PEDV strains. Most assays have been designed to target the genes responsible for encoding one of the structural proteins of the virus S, N or M (Lee, 2015, Diel et al., 2016).

Consideration of the genetic variability is required when designing assays against the spike (S) gene, as it is one of the most variable regions of the PEDV genome and several variant strains in the USA and China contain deletions in this gene (Jarvis et al., 2016). It is therefore important to evaluate assays intended for detection of PEDV by testing against a wide range of samples from diseased pigs to ensure that the assay has high sensitivity and can reliably identify all infected animals. Similarly, samples from virus free populations must be tested to ensure optimal specificity – that is, there are no false positive results. To undertake such evaluations in the face of a disease outbreak, even if it was not due to PEDV, is highly undesirable and could have significantly adverse consequences. Laboratory and regulatory staff and industry leaders need confidence in the diagnostic tools that are available and also know their limitations so that they can be used optimally when undertaking PEDV exclusion testing.

This project aims to address these issues and enhance the national diagnostic and biosecurity capacity. Addition of a PEDV capability to the National Emergency Animal Disease Laboratory Network (LEADDR) is a logical extension of a 'first response' capability, minimising the time to first results and hence maximising chances of successful control.

2. Objectives of the Research Project

1. Assemble a collection of samples from animals across the Australian pig herd
2. 2.Secure suitable kits and PEDV reference samples
3. 3.Evaluate commercially available diagnostic qRT-PCR kits by testing the reference and Australian samples. Compare the performance of the best kit(s) with an 'in house' assay used in a large US diagnostic laboratory
4. 4.Identify the performance characteristics of the preferred diagnostic kit(s) and provide recommendations on their use.

3. Introductory Technical Information

The testing undertaken in this project involves qRT-PCR exclusively. However, no new assays were developed, but rather, the study involved the evaluation of 4 assays that were available from commercial suppliers and 2 assays that had been developed by veterinary diagnostic laboratories in the United States of America (USA). In this report, the commercially available assays are coded numerically (Kits 1-4) and the two published assays are numbers 5 and 6. All assays provided a capability to test for PEDV and all but Assay 5 supported testing for PDCoV.

4. Research Methodology

The qRT-PCR assays that are in use at EMAI and in other state diagnostic laboratories that form the LEADDR network are usually developed and evaluated either *de novo* by one of the laboratories (e.g. AAHL) or are assembled locally based on technical information in scientific publications. Although assays developed in this way depend on local laboratories sourcing and assembling each of the assay components, such assays are usually the most efficient and economical as reagents can be purchased in large quantities for large scale testing or to distribute between laboratories. However, companies that have developed commercially available diagnostic test kits based on ELISA technology (usually for the detection of antibodies) have recently commenced production of kits for qPCR assays. These kits provide the essential reagents and controls in a 'ready to use' format and have been evaluated by the manufacturer. Although more expensive than an 'in house' assembled assay, there is nevertheless some merit in use of these kits, especially under emergency circumstances because the kits are usually optimised and as many steps streamlined as possible. Such kits are appearing on the market for diseases where there is potential for large scale testing, as has occurred for PEDV in the USA in the last 2 years. Therefore, in this project, both commercially available test kits and 'in house' assembled assays will be compared.

4.1 Sample Collection

4.1.1 *Sample size*

As PEDV and PDCoV have not been diagnosed in Australia a low or nil prevalence can be assumed in the target population. Based on a test having a diagnostic specificity (DSp - the proportion of samples from uninfected animals that test negative in an assay) of 95%, a total of 456 negative samples are required (allowing for a 95% confidence and 2% error). Consequently, a target sample size of 500 animals was set.

4.1.2 *Confidentiality*

As a condition of recruitment to the study, co-operating producers were assured of anonymity. No property or owner identification was sought other than the state and region in which the samples were collected. All samples were coded in a manner to ensure additional confidentiality. Any information provided with the samples was held under secure conditions at EMAI on a dedicated PC held by the Principal Investigator and was not be accessible to other personnel.

4.1.3 *Sample collection*

For the collection of 500 representative samples from the Australian pig herd, 20 samples were to be collected from 25 herds. Based on the distribution of sows per state this equated to 3 herds in Western Australia, 4 herds in South Australia, and 6 herds in each Queensland, New South Wales, and Victoria. Provision was made for private pig veterinarians to collect rectal swabs from 10 suckling and 10 weaned piglets with diarrhoea during routine herd health visits. These age groups were selected as they suffer the highest rate or morbidity and mortality from PEDV and PDCoV.

Where it was not possible to collect sufficient rectal swabs, primary producers were approached to provide environmental samples from their herd. Environmental samples consist of a total of 10 swabs of fresh faecal material collected from 1-5 pens for both suckling and weaned piglets.

All samples were collected using sterile cotton tip swabs and were immersed in 3mL of phosphate buffered gelatin saline (PBGS) (viral transport medium) produced at EMAI and sent out as part of a collection kit to the private veterinarian. After collection, samples were held refrigerated and shipped to EMAI chilled. As collection occurred over many months, to minimise any deleterious effects of repeated freezing and thawing, when samples arrived at EMAI, a 1mL aliquot was placed into a cryovial and together with the remaining sample, was stored at -80°C until tested.

4.1.4 *Reference samples*

Reference samples, consisting of rectal swabs or oral fluids, were provided by a large diagnostic laboratory in the USA. These samples had given positive results in either a PEDV or PDCoV assay. On receipt at EMAI they were handled by an experienced senior technician in a DAWR (AQIS) accredited PC3/QC3 containment laboratory and were inactivated by dilution in virus lysis buffer prior to any further testing. These samples were used to provide an assessment of the sensitivity of the assays.

4.2 Laboratory Methodology

4.2.1 *Nucleic acid extraction*

RNA was extracted from samples (environmental and rectal swabs, oral fluids) using the MagMax-96™ Viral RNA Isolation system (Ambion) according to the manufacturer's directions with a Kingfisher-96 magnetic particle handling system (Thermo). To provide sufficient material for testing in all assays, nucleic acids were eluted in 100 µL of elution buffer.

4.2.2 *qRT-PCR assays*

Commercial assays (Assays 1-4) were sourced through the Australian distributors or, in one instance (Kit 3), directly from the USA. Reagents were prepared, and assays run according to manufacturer instructions. Kit instructions varied with regard to sample volume, primers and probes, recommended cycling conditions and analysis parameters. For 'in house' assays, information was provided for a published singleplex PEDV assay (Assay 5) and a published multiplex PDCoV-PEDV assay (Assay 6) by two US veterinary diagnostic laboratories.

Commercial Assays did not provide information regarding primer or probe sequence. Assays 5 and 6 provided primer and probe sequence and were used with a Path-IDTM Multiplex One Step RT-PCR Kit. Assays 1 and 6 contain primers and probes for the multiplex (concurrent) detection and differentiation of PEDV and PDCoV RNA. In contrast Assays 2-4 are triplex assays and also have TGEV detection capabilities in addition to PEDV and PDCoV.

An important element for monitoring the quality of both nucleic acid extraction and the qRT-PCR on an individual sample basis is the inclusion of an internal control. The internal control for Assay 1 is based on endogenous swine RNA present in the sample. This design is based on the expectation that common porcine samples including oral fluids, faeces, faecal swabs, or intestinal contents normally contain sufficient cellular material to act as an internal control. In contrast, the remaining assays, including the 'in house' format used at EMAI, use a synthetic nucleic acid that is added to each sample prior to nucleic acid extraction.

The volume of nucleic acid extract tested in a 25 µl PCR reaction varied from 5-8 µl for different assays. The impact of this is on the outcome is negligible. All plates included a blank control well (nuclease-free water, added after extraction), a negative control (tRNA) used to monitor the efficiency

of extraction and the PCR reaction, and two positive control samples. Positive controls were not provided for Kit 4, but 2 positive samples were included as controls for this assay and for the 2 'in house' assays (5 & 6).

4.2.3 *Thermocycler*

All assays were run in an Applied Biosystems 7500 Fast Real-Time PCR System in a 96 well plate format compatible with the RNA extraction system.

4.3 Data Analysis

4.3.1 *Data collection*

When samples were submitted, to comply with the agreed confidentiality requirements of the project, no property or owner identification was provided other than the state and region in which the samples were collected. No formal laboratory report has been issued to contributors but, subject to approval from APL, collaborators will be provided with a brief summary of the outcome. All data has been coded in a manner to ensure confidentiality is maintained and data is held under secure conditions at EMAI on a dedicated PC held by the Principal Investigator and is not accessible to other personnel.

4.3.2 *Data analysis*

The real time PCR results from all six assays has been interpreted in line with the manufacturer's recommendations. Relative measures of sensitivity and specificity have been calculated using standard statistical methods.

4.3.3 *Internal feedback regarding ease of use*

The technical officer completing the six assays was asked to provide objective feedback on the ease of use of the assays. Ease of use includes instructions provided, resources provided (including positive and internal controls), and complexity of analysis. The Principal Investigator also recorded information relevant to the supply and delivery of commercially sourced kits.

5. Results

5.1 Australian Samples

A total of 485 samples were collected from the Australian pig population. The sample types and distribution of herds are summarised in Table 1 and the demographics related to these samples can be found in Table 2.

Table 1 Australian sample types and geographical distribution

State	Herds sampled	Rectal swabs		Environmental swabs	
		Suckers	*Weaners*	*Suckers*	*Weaners*
Western Australia	4	10	10	30	30
South Australia	6	50	62	0	0
Queensland	7	70	70	0	0
New South Wales	6	40	36	20	20
Victoria	3	24	13	0	0
Sub Total:		194	191	50	50
Grand Total:	28	385		100	

Table 2 Demographics for Australian samples

Average age of suckers	9.2 days
Reported diarrhoea	Mild (62.8%), moderate (31.9%), severe (5.3%)
Average age of weaners	38.1 days
Reported diarrhoea	Mild (86.0%), moderate (10.3%), severe (3.7%).
Average herd size (range)	1708 sows (300-8000)
Average pigs weaned per week (range)	713 pigs (120-3000)

5.2 Relative Specificity of Assays – PEDV and PDCoV

Of the Australian samples collected (n=485) a single environmental sample from a sucker pen was determined to be negative in Assays 2-6 and invalid in Assay 1 only. This sample demonstrated negative PEDV and PDCoV results on the initial extract, repeat testing of the initial extract and follow-up testing of a new extract. However, the results for this sample were deemed to be invalid due to the repeated failure to detect the internal sample control (ISC). Consequently, it was deemed to be inappropriate to include this sample in the overall data analysis and has been excluded from the calculations of assay relative specificity leaving 484 suitable samples.

Rectal swabs (n=385) and environmental samples (n=99) from the Australian pig population all gave negative results for both PEDV and PDCoV across the six PEDV assays and the five PDCoV assays (Table 3).

Table 3 Relative specificity of PEDV and PDCoV assays

Kit	Samples detected negative	Relative Specificity (%)
Assay 1	484	100
Assay 2	484	100
Assay 3	484	100
Assay 4	484	100
Assay 5	484	100
Assay 6	484	100

5.3 Reference Samples

The collection of positive samples were tested in each of the assays for PEDV and in the 5 assays (all but assay 5) that provided a PDCoV capability in duplex format. The results were analysed and interpreted in 2 different ways – firstly by using "cut-off" values from the kit manufacturers (when provided), or as published and, secondly, on the basis of any detectable reactivity in the qRT-PCR assays.

5.3.1 *PEDV qRT-PCR results*

Of the reference samples provided (n=142) a single sample gave negative results in five of the six PEDV RT-PCR assays and all PDCoV assays, after repeat testing of both the original sample and a new nucleic acid extract. Although this sample was classified as a negative result in the "outlying" single assay, it did not meet quality control criteria for the internal control for that assay. Consequently, it was deemed to be invalid for overall data analysis and has been excluded from the calculations of assay relative sensitivity.

Of the 141 reference samples for which data was analysed, 119 samples were positive for PEDV in all six qRT-PCR assays and four samples were negative for PEDV on all six qRT-PCR assays. The four PEDV negative samples have therefore been excluded from the relative sensitivity calculations for PEDV. These samples were positive for PDCoV and were included in the PDCoV evaluation.

Samples with conflicting results (n=9) were subjected to confirmatory testing. Of these 9 samples, Assays 1, 3 and 5 identified all samples as positive, Assay 2 identified 8 as positive Assay 4 detected 7 of the 9 and Assay 6 only detected one of these samples as positive when inconclusive reactors were considered as positive (Table 4).

Table 4 Reference sample results for PEDV assays

Result	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6
Positive	136	135	136	136	124	121
Negative	5	7	6	6	6	14
Inconclusive	0	0	0	0	12	7
Invalid	1	0	0	0	0	0
Total	142	142	142	142	142	142

When the PEDV results were analysed on the basis of any reactivity in the qRT-PCR, the relative sensitivity of most of the kits improved. The distribution of the cycle threshold (Ct) values (reactivity of samples that are not negative) for the assays is shown in Figure 1. Numerically lower Ct values

indicate a higher analytical sensitivity. Figure 2 shows a summary of the same samples, grouped as Ct values <36 or above 36 (a level that is approaching the limit of detection and more likely to give variable results).

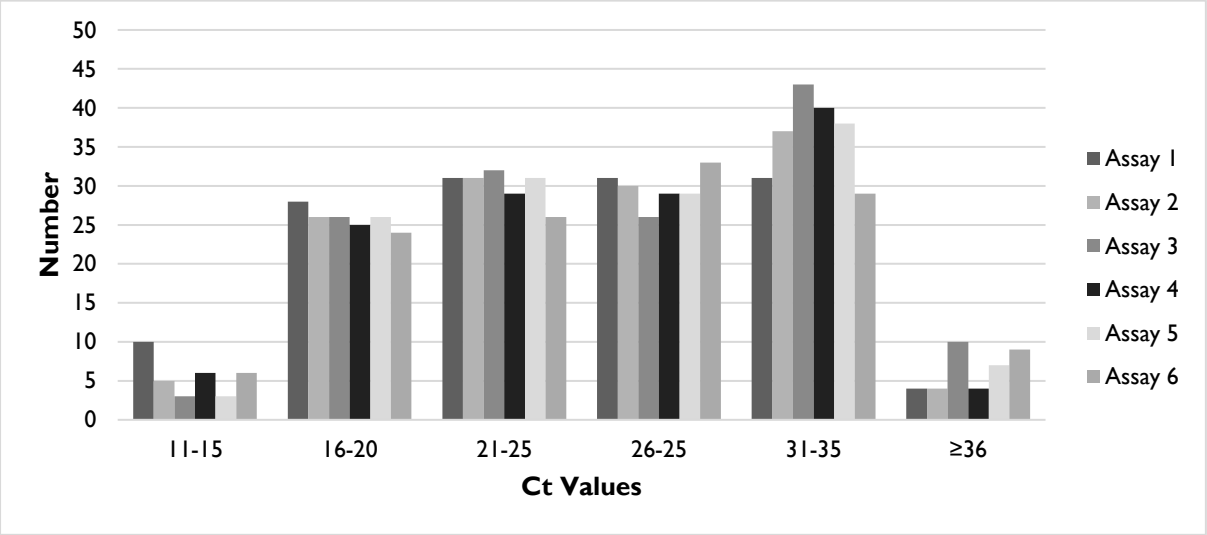

Figure 1 Distribution of cycle-threshold (Ct) values for each PEDV assay

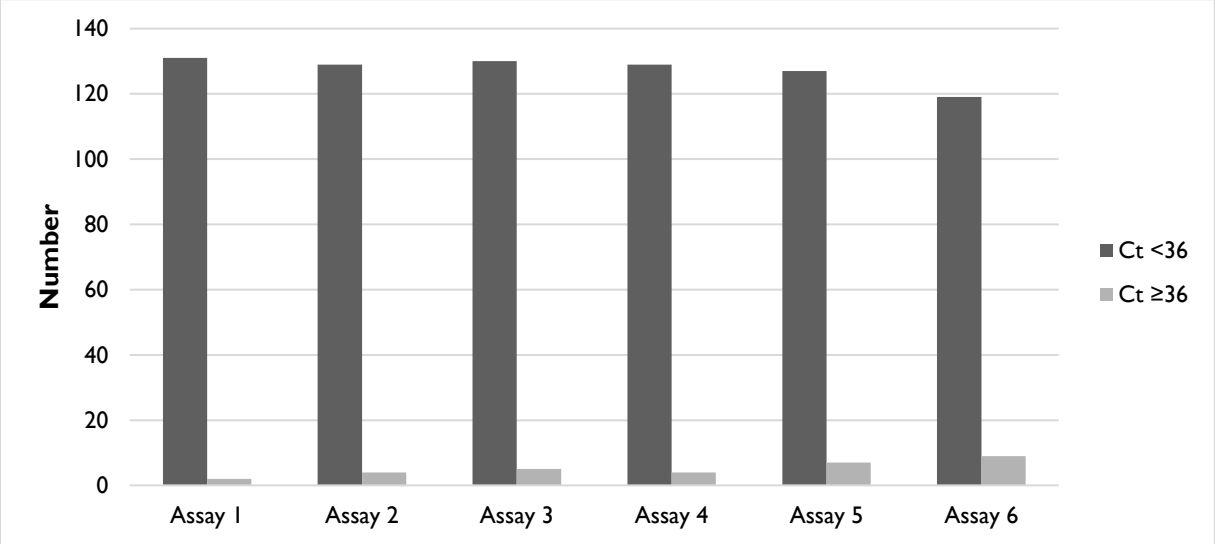

Figure 2 Number of samples giving cycle-threshold (Ct) values above or below 36 for each PEDV assay

5.3.2 *PDCoV qRT-PCR results*

Five assays were included in the assessment of performance as Assay 5 does not offer a PDCoV capability. Of the 141 reference samples 14 samples were positive for PDCoV in all five qRT-PCR assays, with 9 of these samples demonstrating dual infection with PEDV. A total of 116 samples were negative for PDCoV in all five qRT-PCR assays. Samples with conflicting results between the five assays (n=12) were subjected to additional testing, consisting of repeat testing of both the original and a new nucleic acid extract. Assays 1, 3 and 4 identified 6 of the 12 as positive and Assay 6 identified 5 as positive and 4 as inconclusive (Table 5).

Table 5 Reference sample results for PDCoV assays

Result	Assay 1	Assay 2	Assay 3	Assay 4	Assay 6
Positive	21	17	20	20	19
Negative	120	124	118	121	118
Inconclusive	0	0	3	0	4
Total	141	141	141	141	141

When the PDCoV results were analysed on the basis of any reactivity in the qRT-PCR, the relative sensitivity of some of the kits improved, in particular Assay 6. The distribution of the cycle threshold (Ct) values is shown in Figure 3, with a summary of Ct values below or above 36 shown in Figure 4.

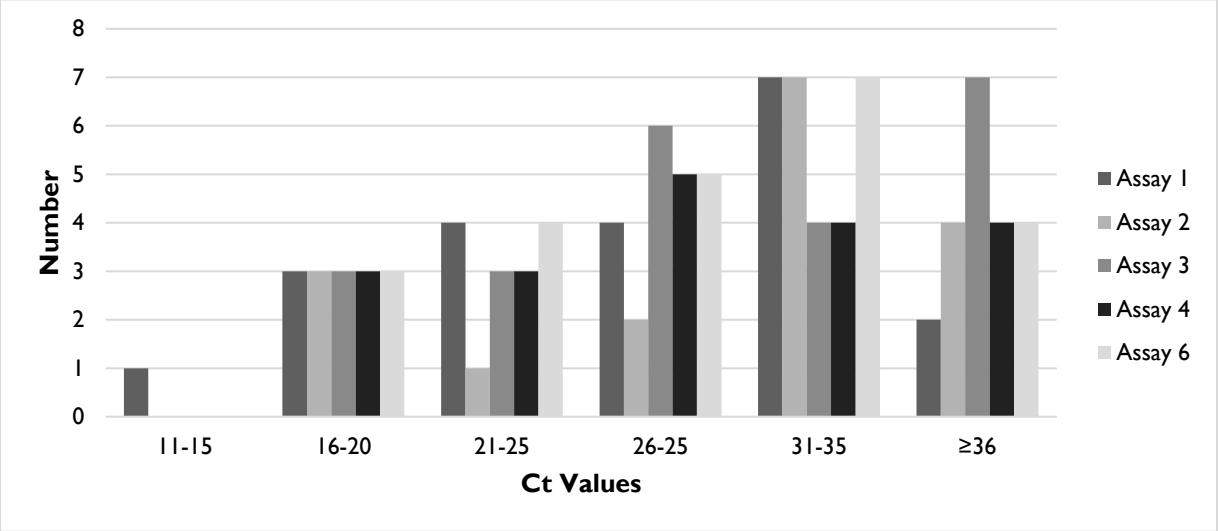

Figure 3 Distribution of cycle-threshold (Ct) values for each PDCoV assay

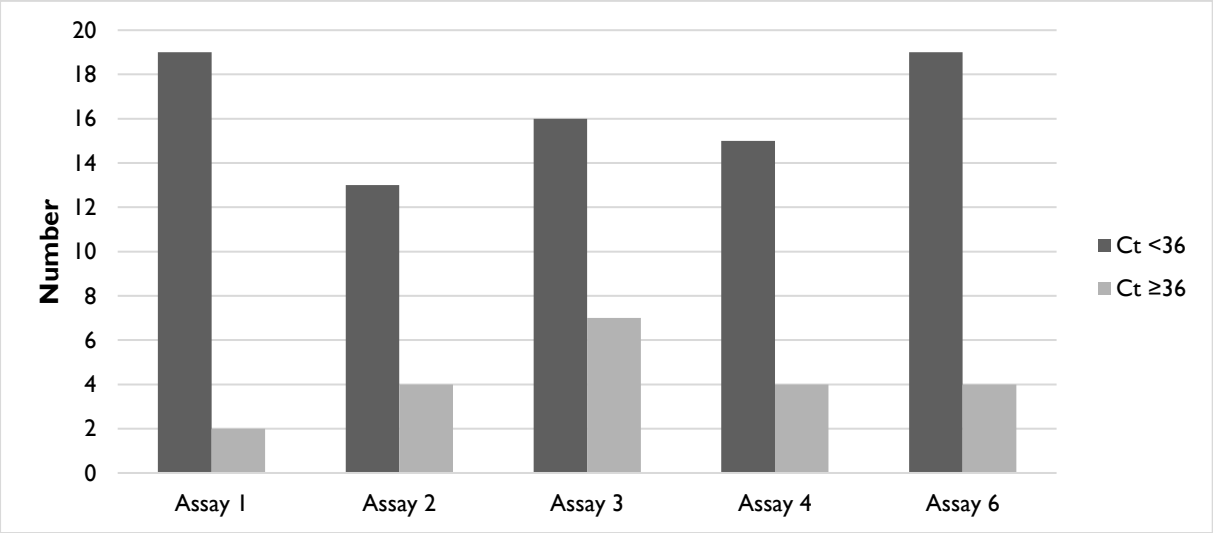

Figure 4 Number of samples giving Ct values above or below 36 for each PDCoV assay

5.4 Relative Sensitivity Calculations

The relative sensitivity was calculated for the PEDV and PDCoV assays using data analysed according to the instructions provided for each assay, and also on the basis of absolute reactivity in each assay (i.e. whether any reactivity was detected or not) (Tables 6 & 7). The confidence intervals for the PDCoV assays were extremely large because of the very small number of samples tested.

Table 6 Relative sensitivity of assays for PEDV – kit interpretation and absolute reactivity

Kit	PEDV – Kit criteria Relative Sensitivity (%) (95% confidence limits)	PEDV – Absolute reactivity Relative Sensitivity (%) (95% confidence limits)
Assay 1	100 (97-100)	100 (97-100)
Assay 2	99.26 (96-100)	99.26 (96-100)
Assay 3	100 (97-100)	100 (97-100)
Assay 4	100 (97-100)	100 (97-100)
Assay 5	91.18 (85-95)	100 (97-100)
Assay 6	88.97 (82-94)	94.12 (89-97)

Table 7 Relative sensitivity of assays for PDCoV – kit interpretation and absolute reactivity

Kit	PDCoV – Kit criteria Relative Sensitivity (%) (95% confidence limits)	PDCoV – Absolute reactivity Relative Sensitivity (%) (95% confidence limits)
Assay 1	84.00 (64-95)	84.00 (64-95)
Assay 2	68.00 (46-85)	68.00 (46-85)
Assay 3	80.00 (59-93)	92.00 (74-99)
Assay 4	80.00 (59-93)	80.00 (59-93)
Assay 5	N/A	N/A
Assay 6	76.00 (55-91)	92.00 (74-99)

5.5 Procurement and performance characteristics

After consideration of assay sensitivity and specificity, the most important aspects for a laboratory to introduce a commercially available assay in kit format include ease of procurement, clarity of both instructions for use and interpretation of results, compatibility with existing equipment, and perhaps similarity of assay method to assays already in use in the laboratory. These considerations have been summarised in Table 8 for the commercial kits and, where relevant, to the 'in house' assays. Of all of the components evaluated, the factors that are considered to be most important are 'off the shelf' supply, ease of use, and cost. Kits 1 and 3 rated highest, though these were the most expensive. Assay 1 was easily sourced and provides clear and concise instructions and interpretation guidelines. Assay 3 provides a suitable alternative to Assay 1 with good relative sensitivity and specificity for PEDV but

does have slightly lower sensitivity for PDCoV. This assay is easily sourced and provides clear and concise instructions and interpretation guidelines. However, this assay requires a more cumbersome analysis than Assay 1 and does not fit into EMAI in-house diagnostic procedures as well as Assay 1. The 'in house' assays would be highly competitive in some situations and the cost is low, but Assay 6 had lower sensitivity while Assay 5 does not provide a capacity to test for PDCoV. However, an experienced laboratory could develop a duplex assay by combining the PDCoV component of Assay 6 with the PEDV reagents from Assay 5.

Table 8 Summary of user assessment of assays

	Assay 1	**Assay 2**	**Assay 3**	**Assay 4**	**Assay 5**	**Assay 6**
Ease of Procurement	✓	✓	✓	✓	✓	✓
Australian Distributor	✓	✓	×	✓	N/A	NA
Short delivery time	✓	✓	✓[5]	××	✓	✓
Off the shelf	✓	✓	✓	✓	✓	✓
Complete kit[1]	✓	✓	✓	×	N/A	N/A
Clear instructions[2]	✓	×	✓	✓	✓	✓
Exogenous internal control	×[4]	✓	✓	✓	N/A	N/A
Sufficient reagents per kit	✓	×	✓	✓	✓	✓
Interpretation of results Included	✓	×	×	×	✓	✓
Analysis easy to perform	✓	××	×	××	✓	✓
Cost[3]	5	3	4	2	1	1
Overall experience	**1**	**5**	**2**	**4**	**3 (1[6])**	**3 (1[6])**

[1] Including all controls

[2] Includes analysis instructions

[3] The numerical scoring is a ranking from lowest (1) to highest (5). Complete qRT-PCR reagent costs range between approximately $6.00-$14 per test for commercial kits; reagents for 'in house' assays cost approximately $3.00 per test. Extraction costs are NOT included. Costs are indicative only and could vary with large scale usage. The cost estimate for Assay 4 is based on reagents supplied plus 'in house' mastermix.

[4] House-keeping target included.

[5] Cold chain could be improved through use of different shipping company.

[6] Components for assays 5 & 6 could be multiplexed and packaged in kit form with all components included (as routinely used at EMAI), changing the overall ranking for laboratories that require an 'off the shelf' kit.

6. Discussion

Molecular based assays, particularly those based on real time PCR technology, have set a new benchmark for the diagnosis of infectious disease. They have many characteristics that are highly suited to the diagnosis of emergency animal diseases. Generally, qRT-PCR assays allow testing of samples without the need to handle or amplify infectious material, have rapid turn-around times, have high analytical sensitivity and specificity, and can be run in a high throughput format that can allow up to 3,000 samples to be tested per day. qPCR assays for new diseases can also be developed and evaluated in much shorter times and with greater confidence than cultural methods. Consequently, it is not surprising that qRT-PCR is the method of choice for the diagnosis of PEDV (Diel et al., 2016).

The principal objective of this study, to evaluate the diagnostic performance of real time PCR assays when testing samples from Australian pigs, was fully achieved. There was no evidence of non-specific reactivity, and because the assays used had a high level of sensitivity to detect PEDV and PDCoV, the negative results conclusively show that the Australian pig population is free of both PEDV and PDCoV. The demonstrated sensitivity and specificity provide confidence in the use of these assays if there is a need to exclude PEDV in a disease incident. The survey design was achieved by targeting high risk groups (nursing and weaner age pigs with diarrhoea) for sample collection. However, the population sampled was unfortunately not quite as representative of the Australian pig population, based on the proportion of sows per state, as planned. Multiple factors resulted in fewer samples being received from Victoria than other states. While this means that the sample was not fully representative of the Australian pig population, it is highly improbable that this has in any way affected the outcome.

The project has identified a range of test options (both commercially available kits and combinations of 'in house' reagents) that will ensure that a national capability should be available at all times. There are assays that can be deployed in both reference laboratories such as AAHL to compliment an established capability or alternatively to equip state laboratories such as those in the LEADDR network to provide a front-line capability. While the 'in house' assays do not achieve the sensitivity of the commercial kits, in practical terms, this is unlikely to seriously impact on diagnostic performance because the RNA levels detected in acutely infected animals should be readily detected by all assays.

The assessment of the capacity of assays to detect PDCoV was a secondary consideration but was included due to the presence of PDCoV initially confounding PEDV diagnosis in the USA. It is important to note that the number of positive PDCoV samples (n=25) included in the reference collection severely limit any rigorous evaluation of the relative sensitivity of these assays. The variations in cut-off criteria for the different assays have some impact on this assessment, as it had for the PEDV assays. While there are some important differences that have been summarised in Table 7, the aspects that caused the greatest concern in this study were the criteria for classification of results as positive or inconclusive. Cycle threshold values are usually used to determine "cut-off" values and provide a useful basis for comparison of relative performance of different assays when testing the same samples. However, as the efficiency of assays can vary near the limit of detection, it may be necessary for one kit to specify a Ct value that differs from another. Very high Ct values can sometimes be generated as a result of non-specific amplification of background nucleic acid, cross contamination or potentially fluorescence artefacts (Caraguel et al., 2011). Consequently, it is important for the manufacturer to provide specific, clear guidelines for the interpretation of the relevant assay. However, as there was no evidence of non-specific reactivity in the current study, there should be less need for a cut-off value. Nevertheless, to put these considerations into perspective, in the face of a PEDV outbreak in a naive population, it would be highly unlikely that samples from acutely infected animals would give

results that present any difficulty for interpretation. The Ct values given by acutely infected animals are likely to be several orders of magnitude below the problematic values that differentiate a weak positive and inconclusive or inconclusive and negative result.

Each commercial assay kit included an 'internal' positive (sample) control that is used to monitor the efficiency of nucleic acid extraction and to assess whether any inhibitors of the PCR are present. An 'in house' internal control used routinely at EMAI was also included for use in the 'in house' assays used by the American laboratories. There are 2 options for these internal controls – a PCR that is designed to detect host (endogenous) nucleic acid, or an exogenous nucleic acid preparation (XIPC) that is added to each sample just prior to nucleic acid extraction. While there are differing preferences for these controls, while the 'spiked' XIPC may appear to be artificial it has clear advantages. Firstly, it is added to each sample in a known concentration and secondly, the XIPC can be designed to be independent of the host species from which the sample was obtained. When an endogenous target is being used, the quantity can vary depending on sample types. Sometimes there can be high (but unknown) concentrations of endogenous nucleic acid at sufficient levels to be still detected in the presence of inhibitors at a level that has abolished detection of the pathogen nucleic acid. In this scenario the assay would meet QC criteria, but a false negative result would be recorded. Conversely, some samples can have extremely low, or in theory, no host nucleic acid. Failure to detect the internal control (combined with a negative result for the pathogen) would result in a correct interpretation that the assay has failed. The use of an exogenous control overcomes these limitations and should provide a clear status of sample quality and reliability of the result for the pathogen. This is demonstrated by a single Australian sample in this study where an invalid outcome was reported for Assay 1 due to the failure to detect the internal control. The PEDV and PDCoV results were negative but there was no way to determine if these negative results were correct. On the other hand, the exogenous control used in all other assays (commercial and 'in house') was reliably detected, confirming that the negative results for the viral RNA were reliable.

When comparing the usability of the assays Kits 1 and 2 provided clear and concise instructions. All kits recommended commencing with "Auto C_T" settings for data analysis. If this did not produce satisfactory results manual thresholds were used to determine the Ct values. Assay 3 and 4 required more time and effort in setting manual thresholds during the analysis when compared to Assay 1 and 2.

7. Implications & Recommendations

In conclusion this study has successfully identified suitable commercial 'kit' assays and also reagents that could be used to establish an 'in house' assay. Although some of the commercial kits are available with a short lead time, and have excellent performance, the considerably higher cost and need for international transport with associated risks of delays could mean that an 'in house' capability may be considered for large scale testing. Of course, the possibility of supplying a large quantity of reagents would also provide significant leverage to negotiate a lower price. Consideration may also be given to holding stock of a preferred commercial kit as well as holding a larger stock of reagents to rapidly assemble an 'in house' assay. As the specifications are known for the 'in house' assays, an option that could be research would be the combination of the PEDV component of Assay 5 with the PDCoV component of Assay 6.

Overall, this project has shown that there are 'ready to use' options to respond to a major disease outbreak. These qRT-PCR assays can support a high throughput capability where results for approximately 100 samples are available within four hours of receipt and up to 3000 samples could be processed per day. Rapid turn-around and provision of results allows the rapid implementation of intervention and control measures which have the potential to reduce the risk of further spread which is paramount for the control of a PEDV outbreak (Lee, 2015, Diel et al., 2016).

8. Intellectual Property

This short project involved a combination of commercially available and published methods. Consequently, there are no elements that could be considered to be intellectual property of a nature that requires protection or worth consideration for commercialisation.

9. Technical Summary

The outcome of this project is evidence that the Australian pig herd is free of both PEDV and PDCoV, viruses that have caused major economic losses in the USA, Europe and parts of eastern Asia. It has shown that there is a range of options to equip Australian laboratories that are involved in the diagnosis of emergency animal diseases.

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11. Publications Arising

As this was a project of less than 6 months duration, there has not yet been any capacity to publish the outcomes of this project. However, an overview of this study was presented at the annual meeting of the Australian Association of Veterinary Laboratory Diagnosticians in Darwin (23-24 November, 2016). Publication of the study results in an appropriate scientific journal are planned.