Australian VETERINARY JOURNAL
THE JOURNAL OF THE AUSTRALIAN VETERINARY ASSOCIATION LTD
PRODUCTION ANIMALS

Comparison of three diagnostic techniques for detection of rotavirus and coronavirus in calf faeces in Australia

MM Izzo,[a]* PD Kirkland,[b] X Gu,[b] Y Lele,[b] AA Gunn[a] and JK House[a]

Objective Compare real-time reverse transcription polymerase chain reaction (qRT-PCR), a commercially available enzyme-linked immunosorbent assay (ELISA) and lateral flow immunochromatography assay (LAT) for the detection of rotavirus and coronavirus in faecal samples collected from diarrhoeic calves.

Design Prospective survey.

Method Samples were tested at two separate facilities using a commercial ELISA and an in-house qRT-PCR. Simple logistic regression was performed to examine the relationship between the two tests. A subset of samples was screened using qRT-PCR, ELISA and a commercial LAT dipstick (132 faecal samples were tested for coronavirus and 122 samples for rotavirus).

Results Of the 586 samples tested, 131 (22.39%) and 468 (79.86%) were positive for coronavirus and group A rotavirus, respectively, using qRT-PCR. The number of samples positive on ELISA for coronavirus and rotavirus was 73 (12.46%) and 225 (38.40%), respectively. Using LAT, 30 (22.73%) and 43 (35.35%) samples were positive for coronavirus and rotavirus, respectively. Simple linear regression revealed a statistically significant ($P < 0.05$) but weak ($r^2 = -0.07$ and -0.40) correlation between the rotavirus/coronavirus qRT-PCR and ELISA, respectively. There was also poor agreement between the LAT and qRT-PCR assays.

Conclusion The sensitivity and specificity of the commercial ELISA and LAT assays evaluated in this study were low compared with qRT-PCR. The low positive and negative predictive values of the assays suggests that they were of limited diagnostic benefit in the population sampled.

Keywords Australia; calves; coronavirus; dairy cattle; diagnostic tests; diarrhoea; enteric pathogens; faeces; rotavirus

Abbreviations Ct, cycling-threshold; ELISA, enzyme-linked immunosorbent assay; LAT, lateral flow immunochromatography; MGB, minor groove binding; ORF1ab, open reading frame 1ab; PBS, phosphate-buffered saline; qRT-PCR, real-time reverse transcription polymerase chain reaction

Aust Vet J 2012;90:122–129 doi: 10.1111/j.1751-0813.2011.00891.x

Neonatal diarrhoea is the most significant cause of morbidity and mortality in dairy calves less than 6 weeks of age.[1] Key variables that affect host immunity, pathogen exposure and, subsequently, the risk of disease include environmental conditions, herd management and nutrition. Disease reflects the culmination of host–pathogen interactions.[2]

*Corresponding author.
[a]Livestock Veterinary Teaching and Research Unit, University of Sydney, 410 Werombi Rd Camden, New South Wales 2570, Australia; matthew.izzo@sydney.edu.au
[b]New South Wales Department of Primary Industries, Elizabeth Macarthur Agricultural Institute, Menangle, NSW, Australia

Generic management strategies are recommended to reduce the risk of neonatal calf diarrhoea, and pathogen-specific interventions such as vaccination or medication may be recommended when a causal relationship is established for specific pathogens. Establishing causality is confounded by pathogen shedding in apparently healthy calves[2] and by the qualitative, but not quantitative, nature of most diagnostic tests used to identify the presence of enteric pathogens in calves.

Rotaviruses and coronaviruses have been identified as the most important viral pathogens involved in the neonatal calf diarrhoea complex.[2,3] Diagnostic techniques that may be used to detect rotaviruses and coronaviruses in faecal samples include virus isolation and electron microscopy, as well as assays to detect viral antigens (latex agglutination and enzyme-linked immunosorbent assay (ELISA)) and viral nucleic acid (such as polymerase chain reaction (PCR)-based assays).[1,4–14] A number of diagnostic tests are used to detect rotaviruses and coronaviruses in calf faecal samples in animal health diagnostic laboratories around Australia. Since diagnostic laboratories moved to full cost-recovery for diseases that are not notifiable, the cost to the producer of diagnostic investigations has increased, leading to a reduction in the use of laboratory assays to support field disease investigations. The development and availability of lateral flow immunochromatography (LAT) dipsticks provides an alternative, affordable, rapid calf-side pathogen detection test for assessment of faecal samples in the field.

Interpretation of ELISA and LAT test results is confounded by limited sensitivity and specificity data. Establishing the sensitivity and specificity of these tests is problematic because it would be largely influenced by the detection limits of the test, the disease prevalence in the sampled population and the number of viral particles present in the samples tested, which is dependent on the timing of sampling during the course of disease and the infective dose.

The development of real-time reverse transcription PCR assays (qRT-PCR) has improved the diagnostic capabilities of large laboratories for the detection of RNA viruses. These assays are sensitive and quantitative diagnostic tests that allow high sample throughput and screening for multiple pathogens. Further, they require less labour, reduce the likelihood of laboratory contamination and are less expensive than conventional gel-based PCR assays. The objective of this study was to evaluate qRT-PCR assays for the detection of bovine rotaviruses and coronaviruses and to investigate the performance of a commercially available ELISA and LAT assay used in Australia for the detection of rotaviruses and coronaviruses in faecal samples from sick calves.

Material and methods

Herd and sample selection

Faecal samples were collected from outbreaks of diarrhoea in dairy and dairy–beef calves under 6 weeks of age. Herds with a minimum of

100 milking cows or rearing a minimum of 15 calves per batch were included in the study. An outbreak of diarrhoea was defined as a minimum of 5% morbidity, with calves exhibiting signs of systemic disease (such as poor appetite, dehydration, decreased mentation and reduced suckle reflex) and pasty to watery faeces. Twelve veterinary practices from the six states of Australia with a large number of dairy herds were instructed on sample selection, sampling technique, storage and transport protocols.

Practitioners were advised to collect 6–10 samples from each farm. Approximately 25 mL of faecal material was collected from the rectum of calves by direct digital stimulation using a new latex glove for each calf. Samples were placed in a sterile container and kept refrigerated until shipping.

Sample processing

Samples were transported on ice from the veterinary clinics to the Livestock Veterinary Teaching and Research Unit, Camden, using an overnight courier service. Faecal samples were refrigerated on arrival and divided into 2 mL aliquots. One aliquot was stored at 4°C until testing with the commercial ELISA and LAT test kits. For the qRT-PCR assays, 0.1 g of undiluted faeces was mixed with 0.9 mL phosphate-buffered saline (PBS) and stored at 4°C until processed at the Elizabeth Macarthur Agricultural Institute. The remaining 2-mL aliquots of faeces were stored at −70°C for further testing if required.

RNA extraction

After low-speed clarification (1500 *g*, 4°C for 10 min) of the 10% suspension of faeces in PBS, 50 µL of the supernatant was used for RNA extraction using a magnetic bead-based system (MagMax 96 Viral RNA, AM 1836 Ambion, Austin, TX, USA) in accordance with the manufacturer's instructions. The magnetic beads were handled and washed and the nucleic acid eluted using a magnetic particle handling system (Kingfisher 96, Thermo, Finland). The nucleic acid was eluted in a final volume of 50 µL and stored frozen at −20°C until tested. Prior to testing by the rotavirus qRT-PCR, the RNA was denatured by heating at 95°C for 5 min.

qRT-PCR assays

Coronavirus. The genome of bovine coronavirus was detected by a qRT-PCR assay that uses a fluorogenic minor groove binding (MGB) probe. Sequence data (Genbank reference FJ 938066) from an Australian strain of coronavirus obtained from a neonatal calf was used to design primers and a probe using Primer Express software version 3 (Applied Biosystems, Foster City, CA, USA). The nucleic acid sequences targeting a segment of open reading frame 1ab (ORF1ab) encoding the polyprotein are as follows:

forward primer (nucleotides 15570–15592): GCG TCC AAA GGC TAT ATT GCT AA; reverse primer (nucleotides 15645–15623): CCC AAC ATT TGG ATT CTG ACA TAA; probe (FAM-MGB) (nucleotides 15585–215602): TGC CTT TCA ACA GGT ATT.

The assay used 20 µL of a commercial qRT-PCR mastermix (AgPath-IDTM One-Step RT-PCR kit, AM1005; Ambion) to which was added 5 µL of extracted RNA. The assay was run on an ABI 7500 Fast thermocycler (Applied Biosystems) for 45 cycles under the cycling conditions recommended by the mastermix manufacturer (reverse transcription at 45°C for 10 min; reverse transcription inactivation/initial denaturation at 95°C for 10 min; amplification for 45 cycles at 95°C for 15 s and 60°C for 45 s). Each assay plate included two negative controls (one negative sample and a no-template control) and two positive controls. Results were analysed using a fixed manual threshold (0.05) and expressed as cycle-threshold (Ct) values. Ct values >40.00 were considered to be negative. The positive controls, derived from a dilution of known positive samples, gave Ct values of approximately 29.00 and 32.00.

During our validation studies, a similar assay was published.[15] The two assays were compared on a collection of 258 of the samples included in this study (data not shown) and were shown to have identical diagnostic sensitivity and specificity, although the analytical sensitivity of the published assay was sometimes slightly lower. The published assay was shown to have an analytical sensitivity of approximately 20 RNA copies/µL and a linear range from 10^1 to 10^9 copies. Our assay was shown to have similar linearity and an analytical sensitivity of approximately 5–10 RNA copies/µL. On the basis of this comparison, our assay was selected as the preferred method for the current study.

Rotavirus. RNA samples were denatured by heating at 95°C for 5 min and tested for groups A and C rotavirus genomes using a modification of the assays described by Logan et al.[16] Our assays used the same volumes, mastermix, cycling conditions and thermocycler as described for the coronavirus qRT-PCR, but used the primers and probes described by Logan et al.[16] Two negative and two positive controls (derived from known rotavirus positive samples) were included on each assay plate. Results were analysed and expressed in the same manner as the coronavirus results.

ELISA testing

A total of 586 faecal samples were tested using a commercial ELISA kit for rotavirus and coronavirus (Pourquier® ELISA Calves Diarrhoea; Institut Pourquier®, Montpellier, France) according to the manufacturer's instructions. Briefly, 50 µL of dilution buffer and then 50 µL of undiluted faeces were plated in triplicate into the wells of a microplate coated with the appropriate antibody. The plate was held at room temperature (approximately 25°C) for 30 min and then washed manually using the wash solution provided. A unique conjugate (one for each of the three pathogens) was then added to the relevant wells for each sample and the plate was held at room temperature for 30 min. Following a final wash, tetramethylbenzidine (TMB) substrate was added to each well and the plate was incubated at room temperature for a further 10 min. A stop solution (0.5 mol/L H_2SO_4) was added and the optical densities were measured at 450 nm using an ELISA plate reader (Labsystems Multiscan Biochromatic; Labsystems, Basingstoke, UK). The ELISA reader optical density data was transformed, according to the manufacturer's recommendations, to calculate the sample to positive (S/P) ratios. Samples with an S/P ratio >7% were deemed to be positive in accordance with the manufacturer's recommendations.

Lateral flow immunochromatography

Faecal samples were tested for coronavirus (n = 132) and rotavirus (n = 122) using LAT dipsticks (Bio-X® Diagnostics; Jemelle, Belgium) according to the manufacturer's instructions. Briefly, a small sample

of faeces was homogenised in a buffer solution and the dipstick was placed into the suspension. A sample was regarded as positive when both the control and positive indicator lines turned red. A sample was regarded as negative when only the control indicator line turned red. A test was regarded null (indicative of a faulty dipstick) when the control indicator line failed to turn red and the sample was retested using another dipstick.

Data analysis

Data were managed in Microsoft Access 2003 (Microsoft Corporation, Redmond, WA, USA) and simple logistic regressions calculated using StatsDirect (StatsDirect Ltd, Cheshire, UK). To investigate the relative classification of samples by qRT-PCR and ELISA according to viral load, each sample was classified by ELISA S/P ratio and qRT-PCR Ct values. A higher ELISA S/P ratio and a lower qRT-PCR Ct value are indicative of higher viral concentrations. The ELISA S/P ratio results were divided into four categories: <7 (negative), 7–25, 25–50 and >50. The Ct values for the qRT-PCR were divided into four categories for each virus: 10–20, 20–30, 30–40 and negative (>40).

Results

Coronavirus and group A rotavirus infection were detected in the faecal samples by all three detection methods (Table 1). Group C rotaviruses were not detected in any of the faecal samples by qRT-PCR, so all reference to rotavirus qRT-PCR results hereafter relate only to group A rotavirus.

ELISA vs qRT-PCR

There was an inverse correlation between the S/P ratio and Ct values for detection of coronaviruses, suggesting agreement ($r^2 = -0.07$) between the different assays. Viral RNA was detected in 18.8% of samples that were negative in the ELISA and a proportion of these samples had low Ct values, suggesting a high viral load (Figure 1).

Table 1. Number of samples positive for the presence of coronavirus and rotavirus by three detection methods

Pathogen	Assay		
	qRT-PCR	ELISA	LAT
Coronavirus	131/586 (22.4%)	73/586 (12.5%)	30/132 (22.7%)
Group A rotavirus	468/586 (79.9%)	225/586 (38.4%)	43/122 (35.4%)
Group C rotavirus	0/586 (0.0%)	–	–

qRT-PCT, real-time reverse transcription polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; LAT, lateral flow immunochromatography.

There was also an inverse correlation between the S/P ratio and Ct values for the rotavirus assays, suggesting agreement ($r^2 = -0.40$) between the different assays. Rotavirus was detected by qRT-PCR in 73.7% of samples that tested negative using the ELISA assay (S/P ratio <7%) (Figure 2). The sensitivity, specificity and positive and negative predictive values for the coronavirus and rotavirus ELISAs compared with qRT-PCR assays are reported in Table 2.

LAT vs qRT-PCR

There was poor agreement between the coronavirus LAT and qRT-PCR assays (Table 2), with only 39 (29.6%) and 30 (22.7%) of the 132 samples tested by both methods positive in the qRT-PCR and LAT assays, respectively. A poor correlation was observed between the Ct values and LAT results, with 5/18 (27.8%) of the low Ct samples (high viral load) and 18/88 (20.5%) of the qRT-PCR-negative samples deemed positive by LAT (Figure 3).

Limited agreement was also seen between the rotavirus qRT-PCR and LAT assays, but a trend was seen between Ct values and LAT results

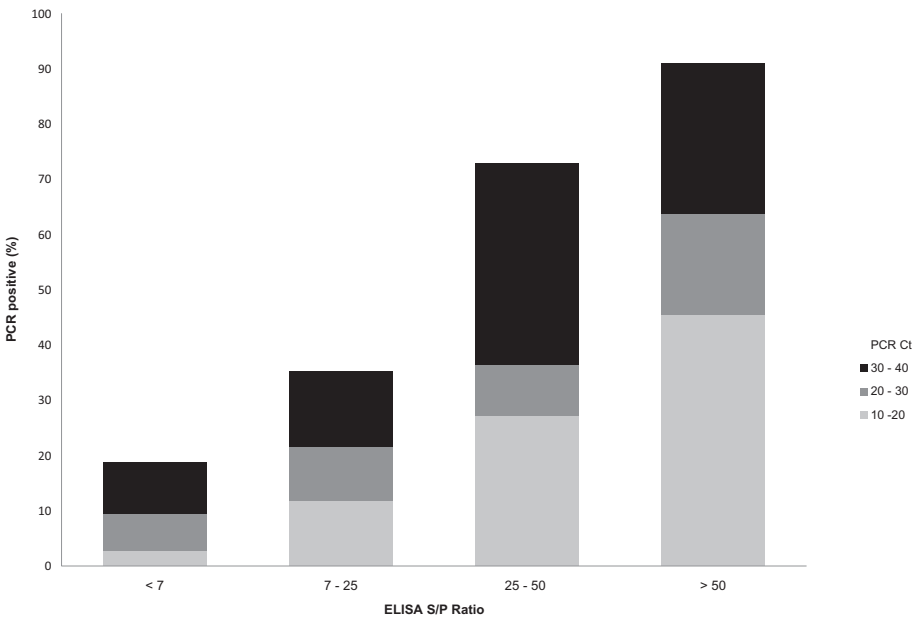

Figure 1. Relationship between enzyme-linked immunosorbent assay (ELISA) S/P ratio and real-time reverse transcription polymerase chain reaction (qRT-PCR) cycle-threshold (Ct) for samples tested for coronavirus. The percentage of samples positive by qRT-PCR is demonstrated for each S/P ratio and the qRT-PCR Ct of the positive samples is stratified according to the figure key.

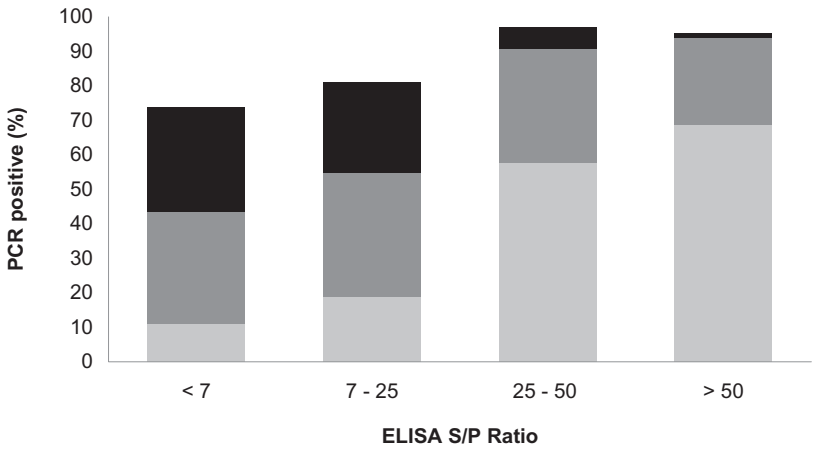

Figure 2. Relationship between enzyme-linked immunosorbent assay (ELISA) S/P ratio and real-time reverse transcription polymerase chain reaction (qRT-PCR) cycle-threshold (Ct) for samples tested for rotavirus. The percentage of samples positive by qRT-PCR is demonstrated for each S/P ratio. The qRT-PCR Ct of the positive samples is stratified as:
10-20
20-30
30-40

Table 2. Agreement among assays for the detection of coronavirus and rotavirus

Assay (reference test)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Coronavirus ELISA				
Coronavirus qRT-PCR	26.7	91.7	48.0	81.3
Coronavirus LAT				
Coronavirus qRT-PCR	28.2	79.6	36.7	72.6
Coronavirus ELISA	33.3	80.4	33.3	80.4
Rotavirus ELISA				
Rotavirus qRT-PCR	44.7	86.4	92.9	28.3
Rotavirus LAT				
Rotavirus qRT-PCR	32.7	46.7	81.4	8.9
Rotavirus ELISA	67.8	95.2	93.0	76.0

PPV, positive predictive value; NPV, negative predictive value; qRT-PCT, real-time reverse transcription polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; LAT, lateral flow immunochromatography.

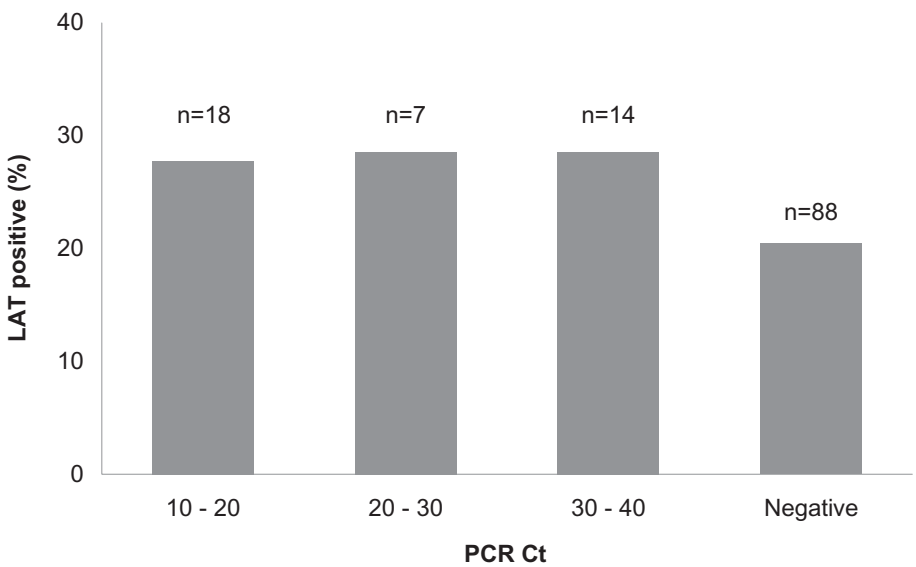

Figure 3. Relationship between lateral flow immunochromatography (LAT) and real-time reverse transcription polymerase chain reaction (qRT-PCR) cycle-threshold (Ct) for samples tested for coronavirus.

PRODUCTION ANIMALS

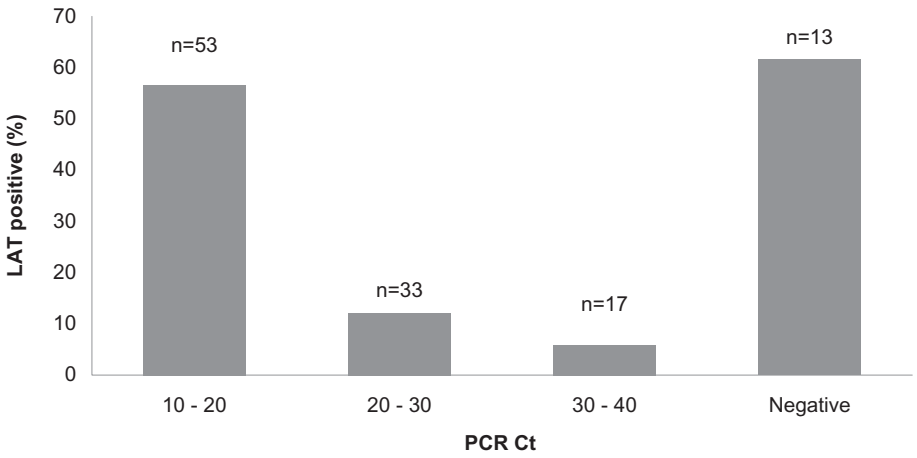

Figure 4. Relationship between lateral flow immunochromatography (LAT) and real-time reverse transcription polymerase chain reaction (qRT-PCR) cycle-threshold (Ct) for samples tested for rotavirus.

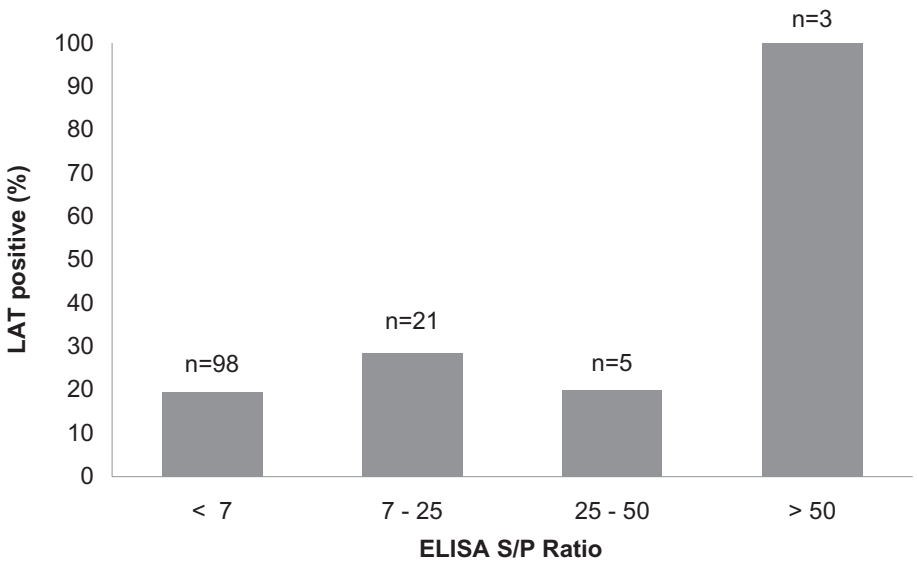

Figure 5. Relationship between lateral flow immunochromatography (LAT) and enzyme-linked immunosorbent assay (ELISA) S/P ratio for samples tested for coronavirus.

(Table 2). However, the qRT-PCR-negative samples included 8/13 (61.54%) samples that were positive by LAT (Figure 4).

LAT vs ELISA

The LAT and ELISA methods both use antibodies to detect viral antigen. However, poor agreement was observed between the two assays for coronavirus detection (Figure 5, Table 2).

The proportion of samples positive for rotaviruses by LAT was high (37/38, 97.4%) for faecal samples that had an S/P ratio >50, but was low (3/18, 16.7%) for ELISA S/P ratios between 7 and 50 (Figure 6). Overall agreement between the two assays was reasonable (Table 2).

Discussion

Establishing a causal relationship between enteric pathogens and outbreaks of diarrhoea in calves is often difficult, because of the propensity for disease to be associated with multiple pathogens and because of the qualitative nature of the diagnostic tests available. The number of organisms shed during enteric infections varies over the course of the disease. A quantitative assay is desirable because it provides an indication of the number of organisms shed and thus a context for interpreting the significance of the finding.

During the acute stage of rotaviral infection, viral shedding in faeces can reach 10^8–10^{12} virions/mL of faeces.[17–19] The pattern of shedding (i.e. peak viral load and duration of shedding) is partially determined by the colostral status of the animal.[20] When calves are infected with rotavirus or coronavirus, the number of organisms shed in the faeces increases over the first couple of days, reaching a peak between days 1–7 post-inoculation. Parreno et al. found a mean duration of rotavirus shedding of 6–10 days, but results were quite variable, with some animals becoming chronic shedders with virus present up to 3 weeks post-inoculation.[20] Experimental studies that examined faecal shedding of coronavirus indicate that coronavirus antigen is able to be detected throughout the period of diarrhoea.[21]

As with many of the enteropathogens, rotavirus and coronavirus can be identified in the faeces of healthy and diseased animals,[2] so a simple

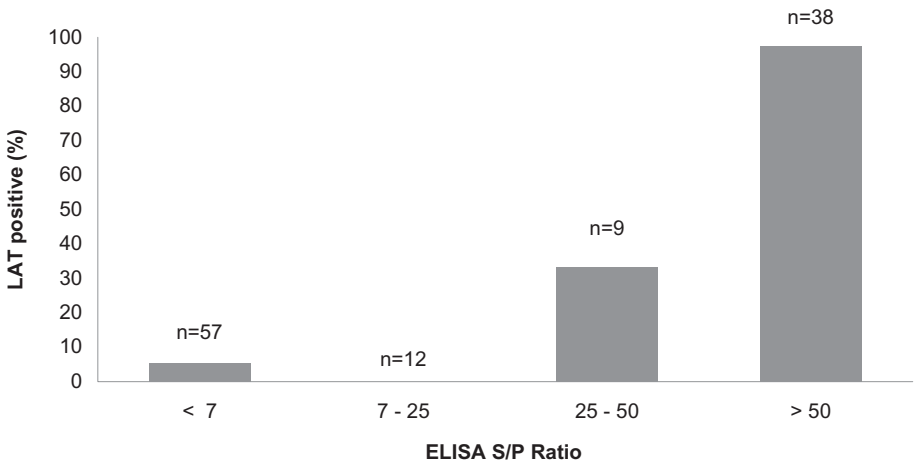

Figure 6. Relationship between lateral flow immunochromatography (LAT) and enzyme-linked immunosorbent assay (ELISA) S/P ratio for samples tested for rotavirus.

dichotomous diagnostic test is insufficient to establish causality. The optimal method of establishing causality is to identify the virus in faeces and to examine affected tissues for classical histopathological changes or presence of the organism at necropsy.

PCR-based assays have been recommended as the gold standard for diagnostic testing for many infectious diseases,[15] with qRT-PCR detection for rotavirus and coronavirus shown to be both highly sensitive and specific when the correct primers and probes are selected.[15,22] These assays have the ability to increase the sensitivity of detection by up to 100-fold when compared with one-step RT-PCR.[23] The two real-time methods that have been used for detection of coronavirus in faeces are a TaqMan assay and a SYBR Green-based assay.[15,24] Detection levels achieved using the TaqMan assay for coronavirus have been in the order of 10^1–10^9 RNA copies and are 10-fold more sensitive than gel-based RT-PCR.[15] The assay using SYBR Green chemistry has similar detection levels, but is pan-reactive and designed to detect any coronavirus, unlike the bovine coronavirus-specific TaqMan assay.[24] In preliminary studies, we found that the SYBR Green-based assay performed erratically and could not be considered as a routine diagnostic test. The assay described by Decaro et al.[15] was compared with our TaqMan-based assay and produced very similar results, but the assay used here sometimes had slightly higher analytical sensitivity (Kirkland, unpubl. data) Guiterrez-Aguierre et al. were the first to describe a real-time TaqMan qRT-PCR for the detection of both human and animal rotaviruses.[22] Both the rotavirus and coronavirus qRT-PCR assays used in the present study were found to be invaluable and provided the capacity for relative quantification of the amount of viral RNA in the samples. Evidence of high viral load in samples tested using qRT-PCR gives the clinician more confidence that the virus identified is likely to be involved in the disease process. This does not exclude the possibility that lower concentrations of RNA may be significant, as the concentration can be influenced by factors such as the stage of disease, the quality of the sample collected and appropriate storage and handling during transport. The RNA extraction and qRT-PCR technologies used also allow large numbers of samples to be tested in a short time and, as a result of the relatively low labour input, can be conducted at much lower cost than conventional RT-PCR.

Although there was a significant association between the ELISA and qRT-PCR for both viruses, the low r^2 values indicate that the results of one assay provided a poor prediction of the results that would be expected with the other. The qRT-PCR assays for rotavirus and coronavirus both detected a higher proportion of positive faecal samples. Similar findings have been reported by others comparing qRT-PCR and ELISA, and were not unexpected.[22] Although it is possible that the discrepant results were related to false positives in the qRT-PCR assays, we consider that this is unlikely, especially considering the high viral load detected in some of the samples. Further, it is known that, for rotavirus, qRT-PCR-positive/ELISA-negative samples in this study did contain viral RNA as demonstrated by further subtyping PCR assays (Kirkland, unpubl. obs.).

A large proportion of samples (73.7%) that were rotavirus qRT-PCR-positive were negative by ELISA. The manufacturer of the ELISA kit evaluated in this study does not supply minimum levels of detection. Detection limits of published ELISAs have been in the order of 10^4–10^6 virions/mL.[14,25,26] The high number of ELISA false-negatives is likely in part to reflect the higher analytical sensitivity of the qRT-PCR assay. Contradicting this argument, a proportion of the ELISA-negative/qRT-PCR-positive samples had low Ct values, indicating a high viral load. However, the different analytes that are detected by these assays should not be overlooked and it is possible that the relatively stable double-stranded RNA of rotavirus may persist under adverse conditions for longer than the protein viral antigens. The rotavirus ELISA assay used in this study targeted the VP7 outer capsule protein. Degradation of the outer virion protein has been described as a cause of false negatives in rotavirus ELISA assays,[27] and stabilisation of the outer capsid can be achieved by including calcium chloride.[28] False-negative results may also reflect the presence of complexing antibodies, high concentrations of faecal material, decreased affinity of detecting antibodies or the presence of proteases.[5,9,21,29,30]

The poor sensitivity of the coronavirus ELISA assay may also in part reflect the limits of detection. However, the poor correlation between the ELISA and qRT-PCR suggests that the variance in the results is likely to reflect other variables. The commercial ELISA evaluated in this study used a polyclonal antibody against the spike protein (S) protein. The viral envelope of coronavirus consists of the nucleocapsid (N) protein and four structural proteins (the haemagglutinin-esterase (HE), S protein, small membrane protein (E) and transmembrane protein (M)).[31–33] The S protein of coronavirus is a common antigen

used for ELISA assays.[12,34] Antigenic variability in the S protein has been observed, because of polymorphism in the S protein, and this variation has been attributed to a single point mutation in the S gene,[21,35] which may lead to altered antibody binding. False negatives may also be related to loss of S protein during degradation of virions or during transport and processing of samples.[36,37]

The development and application of quick, calf-side diagnostic tests is appealing to veterinary practitioners and producers because it avoids the inherent delays associated with shipping samples to diagnostic laboratories. For these tests to provide benefit to livestock producers, it is important that users appreciate each test's limitations. Validation data for commercially available diagnostic tests are often scarce and may be difficult to obtain. According to the manufacturer (Bio-X® Diagnostics; Jemelle, Belgium), the reported sensitivity and specificity of the rotavirus LAT when tested against double-stranded RNA electrophoresis on polyacrylamide gel was 96% and 100%, respectively. The reported sensitivity and specificity of the coronavirus LAT when tested against RT-PCR was 63.6% and 97.4%, respectively. The sensitivity, specificity, positive and negative predictive values of both the rotavirus and coronavirus LAT assays were lower in the present study.

The low positive predictive value of the coronavirus LAT assay (36.7%) reflected a relatively low prevalence of coronavirus in the population sampled, as well as low test sensitivity and specificity when compared with qRT-PCR. The negative predictive value of the coronavirus LAT (72.6%) was considerably higher, but below the negative predictive value of the ELISA (81.3%) when compared with qRT-PCR. The higher prevalence of rotavirus infection in the population sampled provided for a higher positive predictive value (81.4%) of the rotavirus LAT when compared with qRT-PCR, but given the high population prevalence and prior probability of infection, the test provided little additional diagnostic information. The negative predictive value of the rotavirus assay was also extremely low (8.9%) when compared with qRT-PCR, providing essentially no diagnostic value. Recently, Klein et al. evaluated a commercial rotavirus and coronavirus dipstick using faeces from 180 calves (98 with diarrhoea) aged 1–42 days against a RT-PCR assay.[38] The coronavirus assay in that study showed a greater sensitivity (60%), specificity (96.4%), positive (91.3%) and negative predictive values (79.1%) than the coronavirus LAT assay examined in our study. The rotavirus assay in that study also showed a greater sensitivity (71.9%), specificity (95.3%) and negative predictive value (94%), but a lower positive predictive value (76.7%) than the rotavirus LAT assay in our study. Possible reasons may be the increased limit of detection in qRT-PCR compared with normal RT-PCR[23] and a different prevalence of viral pathogens in the two studies (i.e. the prevalence of coronavirus according to RT-PCR in the study of Klein et al. was 38%, whereas it was 22% in our study, and the prevalence of rotavirus in their study was 38.9% compared with 80% in our study).

A possible explanation for the poor performance of the dipstick is that the antigen against which the LAT dipsticks were targeted may have been damaged in transport. It is possible that better results may have been achieved if the dipsticks had been used at the point of sample collection. The application of the tests in the current study was consistent with the use of the dipsticks in a veterinary clinic or diagnostic laboratory.

The sensitivity of the LAT when compared with ELISA for the detection of rotavirus was moderate (67.8%), with very good specificity (95.2%). A previous study comparing the detection of rotavirus using LAT found a sensitivity and specificity of 70% and 100%, respectively, when compared with electron microscopy of 74 faecal samples from calves with acute diarrhoea.[6] Luginbühl et al.[39] also found that the same rotavirus dipstick as that studied by Klein et al.[38] had much lower sensitivity (57%), but the specificity was greater (100%) when compared with an ELISA for the detection of antigens in the faeces of 60 calves. Possible reasons for the difference in our results are that the sample size was much greater in our study and a more sensitive technology was used as the reference assay.

The sensitivity and specificity of both the commercial ELISA and LAT assays evaluated in this study were low compared with qRT-PCR. The low positive and negative predictive values of the assays suggest that they were of limited diagnostic benefit in the population sampled. The qRT-PCR assays offer an alternative diagnostic methodology that is both sensitive and semiquantitative, and thus more informative for clinicians interpreting the significance of a pathogen during disease investigations. Further studies are warranted to develop a better understanding of the clinical relevance of the different levels of viral RNA detected by qRT-PCR assays. When this information becomes available, the higher cost of qRT-PCR assays may be offset by both their superior diagnostic performance and the value of the quantitative information that can be obtained.

Acknowledgments

Funding for the project was provided by Intervet/Schering-Plough Animal Health. Thanks to Laboratory Diagnostics for providing a proportion of the lateral flow dipsticks. The study would not have been possible without the veterinary practices and their clients who provided the samples. The authors are indebted to the staff of the Virology Laboratory at Elizabeth Macarthur Agricultural Institute for technical assistance during this study.

References

1. Takiuchi E, Stipp DT, Alfieri AF, Alfieri AA. Improved detection of bovine coronavirus N gene in faeces of calves infected naturally by a semi-nested PCR assay and an internal control. *J Virol Methods* 2006;131:148–154.
2. Snodgrass D, Terzolo H, Sherwood D et al. Aetiology of diarrhoea in young calves. *Vet Rec* 1986;12:31–34.
3. Quinn P, Markey B, Carter M, Donnelly W, Leonard F. *Veterinary microbiology and microbial diseases*. 1st edn. Wiley-Blackwell, West Sussex, 2002.
4. Alfieri AA, Leite JG, Alfiieri AF, Jiang BG, Gentsch JR. Detection of field isolates of human and animal group C rotavirus by reverse transcription–polymerase chain reaction and digoxigenin-labeled oligonucleotide probes. *J Virol Methods* 1999;83:35–43.
5. Al-Yousif Y, Anderson J, Chard-Bergstrom C et al. Evaluation of a latex agglutination kit (Virogen Rotatest) for detection of bovine rotavirus in fecal samples? *Clin Diagn Lab Immunol* 2001;8:496–498.
6. Al-Yousif Y, Anderson J, Chard-Bergstrom C, Sanjay K. Development, evaluation, and application of lateral-flow immunoassay (immunochromatography) for detection of rotavirus in bovine fecal samples. *Clin Diagn Lab Immunol* 2002;9:723–724.
7. Benfield DA, Stotz JI, Nelson EA, Groon KS. Comparison of a commercial enzyme-linked immunosorbent assay with electron microscopy, fluorescent antibody, and virus isolation for the detection of bovine and porcine rotavirus. *Am J Vet Res* 1984;45:1998–2002.

8. Crouch CF, Raybould TJG, Acres SD. Monoclonal antibody capture enzyme-linked immunosorbent assay for detection of bovine enteric coronavirus. *J Clin Microbiol* 1984;19:388–393.
9. Dea S, Garzon S. Identification of coronaviruses by the use of indirect protein A gold immunelectron microscopy. *J Vet Diagn Invest* 1991;3:297–305.
10. Heckert RA, Saif LJ, Myers GW. Development of protein A-gold immunoelectron microscopy for detection of bovine coronavirus in calves: comparison with ELISA and direct immunofluorescence of nasal epithelial cells. *Vet Microbiol* 1989;19:217–231.
11. Khalili M, Morshedi A, Keyvanfar H, Hemmatzedah F. Detection of bovine coronavirus by RT-PCR in a field study. *Vet Arch* 2006;76:291–296.
12. Reschova S, Pokorova D, Nevorankova Z, Franz J. Monoclonal antibodies to bovine coronavirus and their use in enzymoimmunoanalysis and immunochromatography. *Vet Med* 2001;5:125–131.
13. Sato K, Inaba Y, Tokuhisa S et al. Detection of bovine coronavirus in feces by reversed passive hemagglutination. *Arch Virol* 1984;80:23–31.
14. Schoenthaler SL, Kapil S. Development and applications of a bovine coronavirus antigen detection enzyme-linked immunosorbent assay. *Clin Diagn Lab Immunol* 1999;6:130–132.
15. Decaro N, Elia G, Campolo M et al. Detection of bovine coronavirus using a TaqMan-based real-time RT-PCR assay. *J Virol Methods* 2008;151:167–171.
16. Logan C, O'Leary J, O'Sullivan N. Real-time reverse transcription-PCR for detection of rotavirus and adenovirus as causative agents of acute viral gastroenteritis in children. *J Clin Microbiol* 2006;44:3189–3195.
17. Xu L, Harbour D, McCrae M. The application of polymerase chain reaction to detection of rotaviruses in faeces. *J Virol Methods* 1990;27:29–38.
18. Saif L, Rosen B, Parwani A. Animal Rotaviruses. In: Kapikian A, editor. *Viral infections of the gastrointestinal tact*. Marcel Dekker, New York, 1994:279–367.
19. Theil K. Group A rotaviruses. In: Saif L, Thiel K, editors. *Viral diseases of man and animals*. CRC Press, Boca Raton, 1990:35–72.
20. Parreno V, Bejar C, Vagnozzi A et al. Modulation by colostrum-acquired maternal antibodies of systemic and mucosal antibody responses to rotavirus in calves experimentally challenged with bovine rotavirus. *Vet Immunol Immunopathol* 2004;100:7–24.
21. Crouch CF, Bielefeldt OH, Watts TC, Babiuk LA. Chronic shedding of bovine enteric coronavirus antigen-antibody complexes by clinically normal cows. *J Gen Virol* 1985;66:1489–1500.
22. Gutierrez-Aguirre I, Steyer A, Boben J et al. Sensitive detection of multiple rotavirus genotypes with a single reverse transcription-real-time quantitative PCR assay. *J Clin Microbiol* 2008;46:2547–2554.
23. Munier K, Kibenge F. Analysis of infectious salmon anaemia virus by real-time RT-PCR. *J Virol Methods* 2004;117:37–47.
24. Escutenaire S, Mohamed N, Isaksson M et al. SYBR Green real-time reverse transcription-polymerase chain reaction assay for the generic detection of coronaviruses. *Arch Virol* 2007;152:41–58.
25. Gilchrist M, Bretl T, Moultney K, Knowlton D, Ward R. Comparison of seven kits for detecion of rotavirus in faecal specimens with a sensitive, specific enzyme immunoassay. *Diagn Microbiol Infect Dis* 1987;8:221–228.
26. Rubenstein A, Miller M. Comparison of an enzyme immunoassay with electron microscope procedures for detection of rotavirus. *J Clin Microbiol* 1982;15.
27. Falcone E, Tarantino M, Di Trani L et al. Determination of bovine rotavirus G and P serotypes in Italy by PCR. *J Clin Microbiol* 1999;37:3879–3882.
28. Fitzgerald TA, Browning GF. Increased sensitivity of a rotavirus serotyping enzyme-linked immunosorbent assay by the incorpororation of $CaCl_2$. *J Virol Methods* 1991;33:299–304.
29. Dea S, Tijssen P. Detection of turkey enteric coronavirus by enzyme-linked immunosorbent assay and differentiation from other coronaviruses. *Am J Vet Res* 1989;50:226–231.
30. Maes RK, Grooms DL, Wise AG et al. Evaluation of a human group A rotavirus assay for on-site detection of bovine rotavirus. *J Clin Microbiol* 2003;41:290–294.
31. Holmes KV, Lai MMC. *Coronaviridae: the viruses and their replication*. Lippincott-Raven Publishers, Philadelphia, 1996.
32. Liu LH, Hagglund S, Hakhverdyan M et al. Molecular epidemiology of bovine coronavirus on the basis of comparative analyses of the S gene. *J Clin Microbiol* 2006;44:957–960.
33. Spaan W, Cavanagh D, Horzinek M. Coronaviruses: structure and genome expression. *J Gen Virol* 1988;69:2939–2952.
34. Hasoksuz M, Kayar A, Dodurka T, Ilgaz A. Detection of respiratory and enteric shedding of bovine coronaviruses in cattle in northwestern Turkey. *Acta Vet Hung* 2005;53:137–146.
35. Saif L. Coronavirus immunogens. *Vet Microbiol* 1993;37:285–297.
36. Brandao PE, Gregori F, Richtzenhain LJ et al. Molecular analysis of Brazilian strains of bovine coronavirus (BCoV) reveals a deletion within the hypervariable region of the S1 subunit of the spike glycoprotein also found in human coronavirus OC43. *Arch Virol* 2006;151:1735–1748.
37. Smith DR, Tsunemitsu H, Heckert RA, Saif LJ. Evaluation of two antigen-capture ELISAs using polyclonal or monoclonal antibodies for the detection of bovine coronavirus. *J Vet Diagn Invest* 1996;8:99–105.
38. Klein D, Kern A, Lapan G et al. Evaluation of rapid assays for the detection of bovine coronavirus,rotavirus A and Cryptosporidium parvum in faecal samples of calves. *Vet J* 2009;182:484–486.
39. Luginbühl A, Reitt K, Metzler A et al. Field study about prevalence and diagnostics of diarrhea causing agents in the new-born calf in a Swiss veterinary practice area. *Schweiz Arch Tierhailkd* 2005;147:245–252.

(Accepted for publication 5 May 2011)

ERRATUM

Aust Vet J 2012;90:53

doi: 10.1111/j.1751-0813.2011.00882.x

The laughing vet: anecdotes from a rural practice

RP Knight

The location of Dr Dion Danalis's practice after graduation is incorrect. He established a practice in Swan Hill after graduation, not Mildura, and was there for seven years before returning to Brisbane.