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Journal of Virological Methods 262 (2018) 32–37

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

A convenient colorimetric assay for the quantification of porcine epidemic diarrhea virus and neutralizing antibodies

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ARTICLE INFO

Keywords:
PEDV
Porcine epidemic diarrhea virus
$TCID_{50}$
Virus neutralization
Colorimetric
Plaque assay

ABSTRACT

Neonatal enteritis caused by the porcine epidemic diarrhea virus (PEDV) is an important cause of high mortality and economic losses to the swine industry. Virus neutralization (V/N) assays are commonly requested in diagnostic laboratories for the assessment of protective antibodies. However, the visual assessment of viral cytopathic effects by operators to determine antibody titers or for viral quantification is a tedious, subjective and time-consuming process, especially when high volume testing is involved. To improve the ease of testing, a colorimetric virus neutralization and $TCID_{50}$ assays were developed and validated in this study using (3-(4,5-dimethylthiazol-2-yl) Tr-2,5-diphenyltetrazolium- bromide) (MTT), a colorimetric agent which measures cell viability. The respective conventional assays were used as the gold standards. An OD cut off value of ≤ 0.53, selected by receiver operating characteristics analysis, could distinguish between wells with and without CPE accurately. Performance and reproducibility parameters of the colorimetric assays were comparable to the conventional assays. The described methods can reduce testing time in diagnostic laboratories, while significantly improving current protocols.

1. Introduction

The porcine epidemic diarrhea virus(PEDV) is a highly contagious viral infection of neonatal pigs, characterized by severe diarrhea, vomiting and dehydration. Mortality rates can be as high as 100% in neonatal piglets born to naïve sows (Lin et al., 2014). A member of the alphacoronavirus family, PEDV has been prevalent in Asian countries for over thirty years. It was first detected in the U.S. in 2013 (Chen et al.). Rapid spread of the virus in a naïve population, in conjunction with the high mortality rate resulted in the loss of about 7 million piglets in the first year alone (Schulz and Tonsor, 2015). While PEDV has become enzootic in the U.S. now, it continues to pose a significant economic problem to the pork industry. Therefore, the effective detection and diagnosis of PEDV plays a key role in the control of the disease.

Enzyme linked immunosorbent assays (ELISA) are commonly used for the serological detection of PEDV. However, the quantification of protective antibody responses is often undertaken to determine vaccine-induced protection or herd level immunity against new or related strains, and is assessed by virus neutralization (V/N) tests or fluorescent focus neutralization (FFN) tests in veterinary diagnostic labs (Diel et al., 2016). Both V/N and FFN tests measure the extent of inhibition of viral replication or cytopathic effect (CPE) by the test serum, which in turn depends on the accurate titration of virus stocks used in the assay.

Quantitative reverse transcription PCRs are available for PEDV and used to measure viral nucleic acid content. However, conventional tissue culture infective $dose_{50}$ ($TCID_{50}$) assays are required for the measurement of infective virus. Plaque assays are useful for the quantification of coronaviruses and to distinguish between strains by plaque morphology. However, they are not used frequently for PEDV, perhaps because laboratory culture of PEDV is tedious due to the strict trypsin requirements. Trypsin is required for the cleavage of receptor-bound PEDV S protein and entry into cells (Park et al., 2011). In laboratories with a high volume of testing, the manual and visual evaluation of both the $TCID_{50}$, V/N or plaque neutralization assays are tedious to perform, as they involve the manual examination of numerous wells of 96 well plates or counting of plaques in Petri plates by the operators. Visual assessment of numerous 96 well plates can cause eye and neck strain over long periods of time and thus pose a health hazard. Variation can also be introduced due to differences between operators in scoring the wells as positive or negative, introducing subjectivity in testing.

The goal of this study is to develop a convenient assay to assess PEDV-induced cytopathic effects (CPE)and with applicability to virus $TCID_{50}$ and V/N assays. We have used MTT (3-(4,5-dimethylthiazol-2-

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https://doi.org/10.1016/j.jviromet.2018.09.003
Received 19 June 2018; Received in revised form 2 September 2018; Accepted 9 September 2018
Available online 12 September 2018
0166-0934/

yl) Tr-2,5-diphenyltetrazolium- bromide), a colorimetric agent which measures cell viability. Loss of cell viability is characteristic of virus induced CPE and can be measured by MTT. The reduction of MTT to formazane by cellular dehydrogenases is indicative of the mitochondrial and other enzymatic activity in healthy cells and can be measured as an optical density value with an enzyme linked immunosorbent assay (ELISA) reader (Mosmann, 1983). Therefore, PEDV-infected cells showing CPE are expected to be less efficient in formazane production when compared to healthy cells. Thus, this study is the first description of a colorimetric assay format for a virus neutralization test for a coronavirus. By employing a receiver operating characteristics (ROC) analysis-based cut-off value to distinguish between wells with and without CPE, the optical density (OD) value output from an ELISA reader could be easily used to score wells, reducing the subjectivity and time involved in visual reading. The improved colorimetric methods had a high level of agreement with conventional assays and can significantly reduce the labor involved in the serological testing of PEDV.

2. Materials and methods

2.1. Virus culture

Laboratory culture of PEDV strain CO 2013 (National Veterinary Services Laboratory (NVSL), Ames, IA) was achieved using a monolayer of Vero cells grown to confluence in T-75 flasks, at a multiplicity index (MOI) of 0.1. After infection, the flasks were incubated at 37 °C in 5% CO_2 incubator. Growth media contained Dulbecco's Modified Eagle's Medium (DMEM) (Corning, Manassas, VA), 10 µg/ml Trypsin (Sigma-Aldrich, St. Louis, MO), 7% Tryptose phosphate broth (TPB) (Gibco BRL, Grand Island, NY) and 100 units/mL of penicillin and 100 µg/ml of streptomycin (GE Healthcare Cell Culture, Logan, UT). After 3 h the virus inoculum was removed and fresh growth media was added to the cells. Fresh trypsin was added to the flask every 24 h until the cytopathic effect could be seen at approximately 72 h. The virus culture was harvested with three consecutive freeze-thaw cycles and centrifuged at 2415 x g for 30 min. at 4 °C. The supernatant was distributed in 1 ml aliquots and stored at −80 °C until further use.

2.2. Plaque assay for PEDV quantification

Vero cells were seeded in 6 well plates (Corning) and incubated at 37 °C in a CO_2 incubator until a complete monolayer was formed. Log dilutions of the PEDV virus culture from 10^{-1} to 10^{-6} were prepared in infection media as described above. The monolayers were infected with the 1 ml of diluted culture after washing twice with Hanks Balanced Salt Solution (HBSS) (Corning, Manassas, VA). One well remained as an uninfected cell control. The plates were incubated for 3 h at 37 °C in a CO_2 incubator. The viral inoculum was removed and cells were washed once with HBSS. A 1% solution of low melting agarose (Thermofisher, Waltham, MA) mixed with 2X infection media was overlaid onto the infected monolayers, such that the final concentration of the agarose was 0.5% and the infection media was 1 × . After solidification of the agarose, the plates were incubated in a CO_2 incubator at 37 °C for 72 h. For fixing, 2 ml of 10% buffered formalin (Thermofisher, Waltham, MA), pH 7.0, was added to the each well and incubated for 4 h. Agarose overlays were removed by tapping, and wells were stained with 0.1% crystal violet (Thermo Scientific, Waltham, MA). The virus stock was independently titrated five times by the plaque assay. Plaques were enumerated by two independent operators. As no plaques were visible in wells infected with the 10^{-6} dilution circular, countable plaques which were visible in the wells infected with the 10^{-4} and 10^{-5} logarithmic dilutions were used to calculate the plaque forming units (PFU) by standard methods (Fig. 1) (Liu et al., 2015).

2.3. Virus titration by the conventional $TCID_{50}$ method

The stored aliquots of the PEDV virus culture were titrated by the $TCID_{50}$ method three times, in duplicate, to obtain a total of 6 readings. The assay was set up by the standard method (Schumacher et al., 2016) using logarithmic dilutions of the culture ranging from -1 to -8, in 96 well plates. Six wells were used for each dilution. The process for virus culture was essentially as described above, except 96 well plates were used and 100 µl of each log dilution was plated per well. Cell controls incubated with growth media to rule out any other causes of toxicity and undiluted virus controls were included in each plate. The presence of CPE in each well was scored by visual microscopic examination, by two independent operators and titers calculated by the Spearman and Karber method (Ramakrishnan, 2016).

2.4. Colorimetric MTT_{50} assay

On completion of the visual reading, the supernatant in each well was removed by aspiration. A solution of 0.5 mg/ml (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium- bromide) (Thermo Scientific, Waltham, MA) (MTT) was prepared in sterile phosphate buffered saline (PBS), pH 7.0, and added to each well in 100 µl volumes. The plates were incubated in a CO_2 incubator for 4 h to reduce MTT into formazan. The supernatant from each well was carefully removed without disturbing the cells by aspiration. To solubilize the intracellular formazan, 100 µl dimethyl sulfoxide (DMSO) (Thermo Scientific, Waltham, MA

), was added to the each well, mixed well by vigorous pipetting, and incubated for 5 min. at 37 °C. Plates were read at 570 nm in an ELISA microplate reader (Biotek, Winooski, VT) to obtain optical density (OD) readings.

2.5. Receiver operating characteristics (ROC) analysis

To determine the most optimal OD cut-off value to distinguish between wells with and without virus-induced CPE, the readings obtained by visual assessment for the $TCID_{50}$ assay were used as the gold standard for analysis, since the $TCID_{50}$ is well-established as a standard method for viral quantification.

Dichotomized values of the visual readings from $TCID_{50}$ assays were analyzed against the OD value read outs obtained from the ELISA plate reader, after addition of MTT to the plates. The sensitivity (Y axis) was plotted against the 100-specificity (X axis) for different cut-off points of the OD values, to obtain the predicted sensitivity and specificity at each point. The area under the curve was used to determine the accuracy of distinguishing between positive and negative wells, with 100% values representing perfect discrimination (Fig. 3). The sensitivity and specificity of differentiating wells with and without CPE, and the positive and negative predicative values at the software generated OD cutoff value off were obtained using a commercial statistical software package (MedCalc software, MedCalc Inc., Ostend, Belgium). Agreement between wells scored visually and by the OD value was assessed by the Kappa statistic.

3. Conventional and colorimetric virus neutralization (V/N) assays

A standard V/N assay format (Chen et al., 2016) was optimized using control sera purchased from the National Veterinary Services Laboratory (NVSL), Ames, IA. Briefly, 10 doubling dilutions of the positive and negative control sera, starting at a 1:2 dilution, was prepared in DMEM and incubated with an equal volume (50 µl) of 10^3 $TCID_{50}$ of the PEDV virus stock in 96 well U bottom plates for one hour at 37 °C. After washing the wells twice with HBSS, the serum and PEDV mixture was transferred to 70% confluent Vero cells monolayers. The plates were incubated at 37 °C in a CO_2 incubator for 3 h. The inoculum was

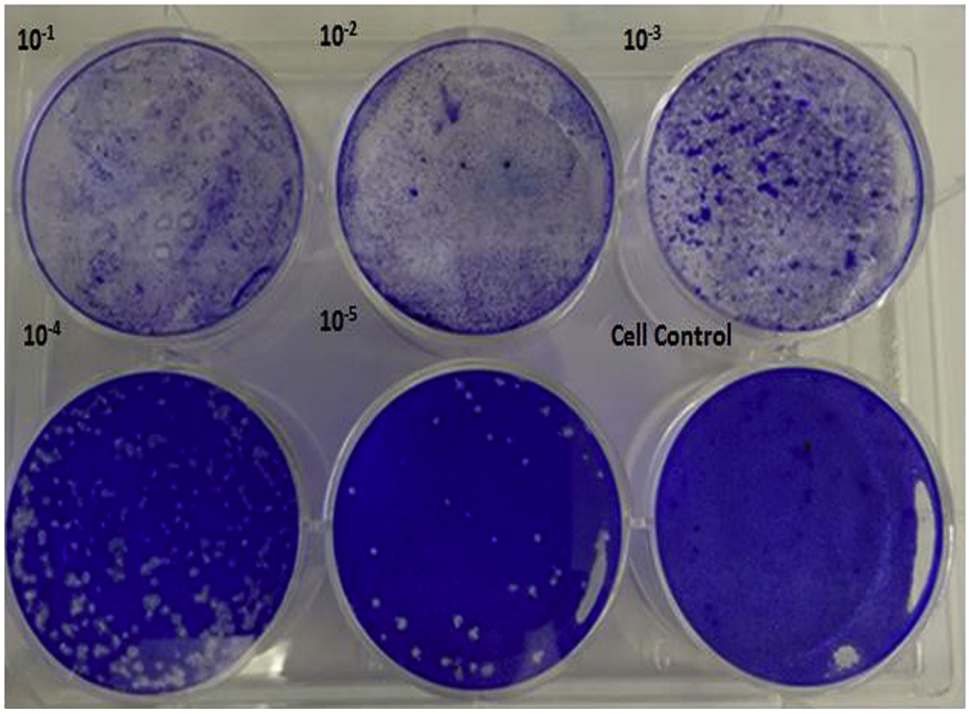

Fig. 1. Plaque assay of the PEDV virus culture: Representative image of the PEDV plaque assay showing a complete loss of the cell sheet in the lower dilutions and distinct viral plaques in the 10^{-4} and 10^{-5} dilutions. The cell control shows an intact cell sheet.

removed after the initial incubation and replaced with infection media, as described in the virus culture method. Six wells each were maintained as either uninfected controls or virus controls. After incubation for 48 h, the plates were read visually to assess inhibition of CPE by the test serum. A 90% inhibition of viral replication was considered the end point to assign the V/N titer (Thomas et al., 2015).

Thereafter, to adapt the assay to the colorimetric format, MTT was added to the plates as described above. Optical density values corresponding to the visual readings were obtained by reading the plates in an ELISA reader. For the colorimetric assay, wells were scored as positive or negative based on the OD cutoff value previously determined by Receiver Operating Characteristics (ROC) analysis. The colorimetric V/N titer was determined as the lowest dilution at which the wells had an OD value ≤ the cut off value. The agreement between the conventional and colorimetric tests were assessed by comparison of the titers obtained by the two methods. To assess reproducibility in terms of inter and intraassay variation, the positive and negative controls were titrated by the conventional and colorimetric V/N assays in triplicate on two separate occasions indicated as replicate 1 and 2 with values from 1 to 6 in Table 1. Sera from pigs infected with the other commonly prevalent viruses like porcine circovirus strain 2 (PCV2), porcine reproductive and respiratory disease syndrome virus (PRRSV) were used to test for specificity.

Table 1
Inter and intra-assay variation of the colorimetric PEDV virus neutralization assay*.

	Replicate 1#			Replicate 2#		
	1	2	3	4	5	6
Negative						
Visual	1:2	> 1:2	> 1:2	> 1:2	> 1:2	> 1:2
Colorimetric*	1:2	> 1:2	> 1:2	> 1:2	> 1:2	> 1:2
Positive						
Visual	< 1:1024	< 1:1024	< 1:1024	1:256	1:512	< 1:1024
Colorimetric	< 1:1024	< 1:1024	< 1:1024	1:256	1:512	< 1:1024

* A cut-off value of ≤ 0.53 OD was used to distinguish between positive and negative wells for the colorimetric assay.
# Two independent assays with triplicate values each.

Sera were selected randomly from six PEDV-suspect farms, submitted to the serology section of the South Dakota Animal Disease Research and Diagnostic Laboratory (SDSU-ADRDL) to validate the colorimetric V/N assay on field samples. The samples were assessed using a fully standardized PEDV N protein-based ELISA protocol, routinely used at the S. Dakota State University Veterinary Diagnostic Laboratory (Okda et al.). Seropositive (N = 35) and negative sera (N = 15) (total N = 50) were randomly selected based on the ELISA. Reactivity of the selected samples to PEDV further confirmed by a standard immuno-fluorescence assay as described before (Song et al., 2016). The selected samples were tested by both the conventional and colorimetric V/N assays in 2 independent assays (Table 2).

All experimental protocols were carried out in compliance with the Institutional Biosafety Committee of North Dakota State University and South Dakota State University.

3.1. Data analysis

The agreement between the $TCID_{50}$ assay, plaque assay and MTT_{50} was assessed by the Spearman's rank coefficient or kappa statistic. The agreement between the conventional V/N tests and the MTT V/N assay was assessed by the Spearman's rank coefficient. The $\log_2$ values of the V/N titers were analyzed by a Student's *t*-test (Chen et al., 2016). All data analysis was carried out using commercial statistical software (MedCalc software, MedCalc Inc., Ostend, Belgium) and p values < 0.05 were considered significant.

4. Results

To ensure accuracy initial quantification of the virus stock was first measured both by plaque assay, where growth of the virus could be visualized as plaques, and $TCID_{50}$, where virus replication could be visualized as CPE. As expected, wells with a higher concentration of virus showed a complete loss of the cell sheet in the plaque assay. Distinct, countable plaques were detected in the 10^{-4} and 10^{-5}

Table 2
Assessment of field samples by the conventional and colorimetric V/ N assays*.

Sample	Replicate 1		Replicate 2	
	Colorimetric	Conventional	Colorimetric	Conventional
1	1:32	1:16	1:32	1:16
2	1:16	1:16	1:16	1:16
3	1:8	1:8	1:16	1:16
4	1:64	1:64	1:128	1:128
5	1:64	1:64	1:64	1:64
6	1:32	1:32	1:64	1:64
7	1:16	1:16	1:8	1:8
8	1:8	1:8	1:8	1:8
9	1:32	1:32	1:32	1:32
10	1:64	1:64	1:64	1:64
11	1:8	1:8	1:16	1:8
12	1:16	1:8	1:8	1:8
13	1:32	1:32	1:32	1:32
14	1:16	1:16	1:16	1:16
15	1:32	1:32	1:16	1:16
16	1:128	1:128	1:64	1:128
17	1:128	1:128	1:64	1:128
18	1:64	1:64	1:64	1:64
19	1:128	1:128	1:128	1:128
20	1:32	1:32	1:32	1:32
21	1:32	1:32	1:32	1:32
22	1:64	1:32	1:32	1:32
23	1:256	1:128	1:128	1:128
24	1:128	1:128	1:128	1:128
25	1:64	1:64	1:64	1:64
26	1:128	1:128	1:128	1:128
27	1:256	1:256	1:256	1:256
28	1:64	1:64	1:128	1:128
29	1:128	1:128	1:128	1:128
30	1:64	1:64	1:128	1:128
31	1:256	1:256	1:256	1:256
32	1:256	1:256	1:256	1:256
33	1:256	1:256	1:256	1:256
34	1:512	1:512	1:1024	1:1024
35	1:32	1:16	1:32	1:32

* A cut-off value of ≤0.53 was used to distinguish between positive and negative wells.

dilutions (Fig. 1). The average titer of the virus stock based on the plaque assay was $10^{6.05 \pm 0.43}$ plaque forming units (PFU) while the mean titer obtained by $TCID_{50}$ titrations was $10^{4.55 \pm 0.21}$. The agreement between the $TCID_{50}$ and the plaque assay as measured by the Spearman's rank coefficient was 0.975 with a p value of 0.005. The 95% confidence interval for rho was 0.660–0.998. The cell controls remained uninfected while the virus controls showed clear CPE, for the duration of the incubation. As expected for the MTT_{50} assay, the OD values obtained after addition of MTT were low for wells with higher

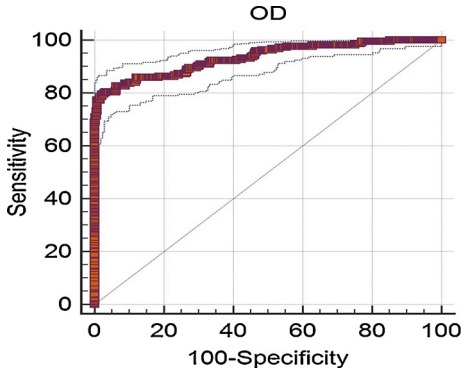

Fig. 3. Receiver Operating Characteristics (ROC) analysis: Dichotomized values for the visual readouts from 96 well plates of the $TCID_{50}$ assays were compared with the OD values obtained from the MTT assay using ROC analysis tool in the MedCalc Inc. software, to obtain a cut-off value to distinguish between positive and negative wells. The true positive rate (sensitivity) and the false positive rate (100-specificity) were plotted at the Y and X axis respectively at different cutoff points for the OD values. The curve obtained is indicative of the sensitivity and specificity of the assay at a given cutoff point. The top left corner, representing the area under the curve (AUC) or accuracy of the test, was 0.931. At the selected cutoff of ≥ 0.53 the specificity was 95%, while the sensitivity was 81% with a p value of < 0.0001.

concentrations of virus, and vice versa, reflective of low cell viability due to viral infection. The uninfected cell controls had an average OD value of 1.10. The relationship between decreasing concentrations of virus in the logarithmic viral dilutions and increase in the OD value on the MTT assay was linear with the equation y = 0.1496x + 0.0821, R^2 = 0.916, indicating specific detection of viral replication by the MTT assay (Fig. 2) (Ramamoorthy and Singh, 2018).

Using dichotomized values from the visual reading as the gold standard for ROC analysis, an OD value of ≤ 0.53 was selected as the optimal cutoff to distinguish between wells with and without CPE. The area under the curve was 0.933 with a Z statistic p value of < 0.0001 (Fig. 3) (Ramamoorthy and Singh, 2018). The area under the curve represents the accuracy of the test, with a value of 1.0 representing a perfect test. The p value is the statistical probability that the test can accurately distinguish between positive and negative samples. At this software generated cutoff, the positive and negative predictive values were both 90% respectively and the specificity was 95%, while the sensitivity was 81%. The kappa statistic for the agreement between the visual and MTT read outs was 0.83, with a 95% confidence interval of 0.780 to 0.874. These values indicated a good agreement between the tests.

When samples were tested by both the conventional V/N and MTT

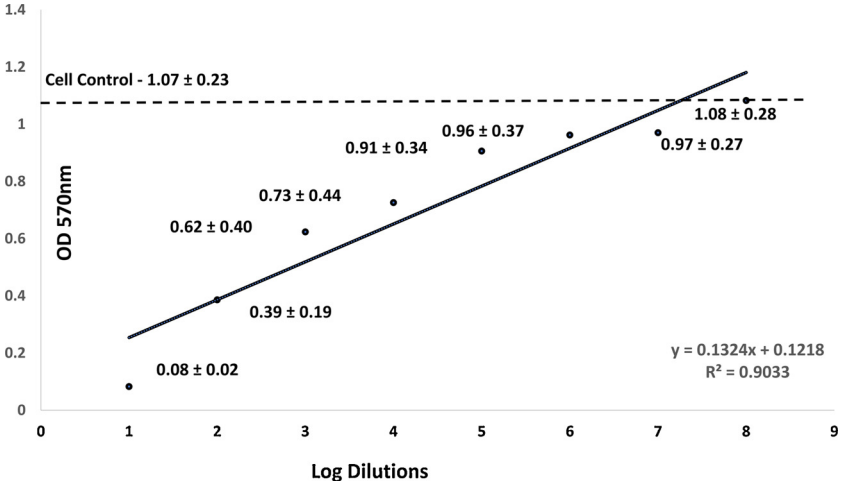

Fig. 2. Linear relationship between the logarithmic viral dilutions and OD values: The average OD values for each logarithmic dilution obtained from the readout of the MTT assay plotted against the logarithmic dilution factor. The relationship was linear between the 10^{-1} and 10^{-5} log dilutions. The dashed horizontal line indicates the average OD value of the cell controls.

V/N assays determine agreement between the tests, the conventional V/N assay performed as expected since the commercial positive control sample showed high V/N titers while the negative control did not (Table 1) (Ramamoorthy and Singh, 2018). Following visual assessment of the 96 well plates, MTT was added to the wells and OD values obtained as the read out from the ELISA plate reader. The colorimetric V/N titer was determined as the lowest dilution at which the wells had an OD value ≤ the cut off value of 0.53. There was complete agreement between the conventional and MTT V/N assays across the triplicate testing in two replicate assays, indicating high reproducibility with no measured inter or intra assay variation between the two compared tests (Table 1) (Ramamoorthy and Singh, 2018). There was no statistically significant difference between the groups as assessed by a Student's *t*-test ($p = 0.71$). Two-fold differences were noted for the positive control sample between two titrations of replicate 2. This difference is generally accepted to be within the detection limits for V/N assays (Stephenson et al., 2009) or can be attributed to operator error but did not affect agreement between the colorimetric and conventional V/N tests. Similarly, high levels of consistency between the conventional and colorimetric V/N's was evident in the field samples tested with only 4 samples showing a two-fold inter assay variation (Table 2) (Ramamoorthy and Singh, 2018). The negative field samples tested had a titer of 1:2 or > 1:2 in both assays. To test non-specific samples by the V/N assay, serum samples from porcine circovirus strain 2 (PCV2), porcine reproductive and respiratory syndrome virus, porcine respiratory coronavirus (PRCV), transmissible gastro-enteritis virus (TGEV) or porcine delta coronavirus (PDCoV) infected pigs were tested by conventional and colorimetric V/N assays. The PCV2 and PRRSV specific sera were obtained from a previous study (Ramamoorthy et al., 2011). A pooled sample of sera (N = 5) collected at 28 days post infection was tested in duplicate. The PRCV, PDCoV or TGEV- specific sera were gnotobiotic pig derived antisera provided by Dr. Linda Saif, Ohio State University and were also tested in duplicate. Similar to other studies (Lin et al., 2015), detectable neutralization of PEDV with the non-specific controls was not observed in this study.

5. Discussion

While quantitative PCR based methods are convenient and commonly used for the detection of active PEDV infections, virus quantification methods such as $TCID_{50}$ titrations are required for measurement of infective virus and has application in the study of pathogenesis, immunity, vaccine development, and the isolation and propagation of field strains. Similarly, while ELISA's (Song et al., 2016) are useful in assessing the serological status of animals, the measurement of protective, neutralizing antibody responses are the method of choice for determining vaccine efficacy and the level of protection or cross-protection in a herd. Colorimetric assays, based on MTT, have been previously developed for the titration of other viruses such as parvoviruses (Heldt et al., 2006) and picorna viruses (Andersson et al., 2005). However, this study is the first description of adaption of the method for V/N assays and for the quantification of PEDV, a coronavirus. The primary advantage of the colorimetric assay is that it can be easily adapted to a high throughput format for use in veterinary diagnostic labs. It also eliminates the subjectivity, labor and time involved in visual examination of $TCID_{50}$ or virus neutralization assays for PEDV.

Culture of PEDV in Vero cells typically results in the production of virus stocks with titers that range from about 10^3 to 10^6 $TCID_{50}$, with the titers increasing over passages (Chen et al., 2014; Hofmann and Wyler, 1989). Similar to previously described findings, a titer $10^{4.5}$ $TCID_{50}$ was obtained for the virus culture used for optimization of the MTT_{50} colorimetric assay. Few other published studies describe the use of the plaque method for PEDV quantification, probably because of the tedious nature and longer turnaround time of the assay (Hofmann and Wyler, 1989; Oka et al., 2014; Zhang et al., 2015). In this study, a titer of 10^6 PFU was obtained in the plaque assay for the same culture, which deviates from the general rule of thumb suggested by ATCC that PFU = 0.7x $TCID_{50}$ (ATCC, 2012). However, virus titrations are prone to biological variation, in addition to the inherent limitations of serial dilution methods and possible differences between operators in the assessment of CPE or plaque enumeration. Hence, replication of assays was carried out to ensure accuracy. The differences in the methodology for $TCID_{50}$ and plaque assays such as the agar overlay, staining to visualize plaques and the acceptable limits for countable plaques are other sources of variation between $TCID_{50}$ and plaque assays. Therefore, variation between $TCID_{50}$ and plaque assays may deviate from the rule of thumb for different viruses and culture systems. Similar to another study, comparing a fluorescent focus assay with the plaque assay for PEDV (Cruz and Shin, 2007), the agreement between $TCID_{50}$ and plaque assays for PEDV in this study was excellent, with a correlation coefficient of 0.975.

Two other studies describe the successful use of MTT to colorimetrically quantify viral CPE, even reducing virus titration time for parvoviruses from 7 to 2 days (Andersson et al., 2005; Heldt et al., 2006). In these studies, the OD value to corresponding to the 50% end point was either applied directly (Andersson et al., 2005) or calculated as 50% of the OD of the negative control (Heldt et al., 2006). Similar to Heldt et al. (2006) where half the mean value of the negative controls was used as the cutoff to distinguish positive and negative wells, in this study, the mean OD of the uninfected cell controls was 1.1 and the selected cut off value of 0.53 was approximately half the mean value of the uninfected cell controls. However, unlike Andersson et al. (2005) who used half the mean OD of the 50% end point as their cut-off value, using 0.35 or half of the average OD value of the 50% end point (0.7) in this study as the cut-off would have reduced the sensitivity and specificity of the assay significantly. To improve accuracy, and obtain a standardized OD cut off value with reliable sensitivity and specificity parameters, that could be applied across test batches in the same laboratory, we used ROC analysis as the tool of choice. The selected OD cut-off value to distinguish between wells with and without CPE, when applied to scoring wells and the calculation of MTT_{50} values using the Spearman and Karber formula, produced excellent agreement between the conventical and MTT-based assays. While the agreement between the colorimetric and conventional tests was also high in the other two studies, detailed statistical analysis was not available in these publications to compare our approach of using ROC analysis versus directly applying an OD value to obtain the 50% end point.

With improved understanding of the mechanisms of action of MTT, it is now known that MTT reduction occurs not only in the mitochondrion but also by the activity of cellular oxidoreductases throughout a cell (Berridge et al., 2005). In addition, oxidative stress, glycolysis and factors which influence metabolism or energy also influence MTT reduction. Since MTT measures several cellular enzymatic activities simultaneously, its reduction can be significantly influenced by changes in experimental parameters (Stepanenko and Dmitrenko, 2015). Further, comparison of the MTT and trypan blue assays for measuring adenovirus replication showed differences between the two assays at early time points (Tollefson et al., 1996; van den Hengel et al., 2011), with reduction of MTT occurring even before the onset of cell death (Tollefson et al., 1996). Hence, it is important to validate of the cut-off point with the specific culture conditions of each laboratory to avoid inter-laboratory variation and potential under/overestimation of cell viability.

Virus neutralization tests for PEDV often used in the field to determine the level of protective antibodies, as a follow up for vaccination or in herds where animals show clinical signs (Okda et al., 2015) and are also tedious to assess visually, especially for high volume testing. This study is the first to describe methods for a colorimetric V/N tests for PEDV which can significantly reduce the labor effort in performing this test. Conventional virus neutralization tests for PEDV are well established (Chen et al., 2016; Cruz and Shin, 2007; Okda et al., 2015) and the primary goal of this study was to compare the conventional and

colorimetric V/N's. Obtaining large numbers of quality samples from experimentally infected animals at the time this study was conducted was a challenge as PEDV was relatively new in the U.S. However, the design of this study meets the WHO guidelines stating that analytical validation is considered sufficient for incremental or adjunct tests (2017).

In general, it is accepted that there is good agreement between spike-protein specific Ab levels and V/N titers (Paudel et al., 2014) but it is not clear if there is a direct correlation between the PEDV-specific IgG levels as assessed by the N-protein ELISA used in this study (Okda et al., 2015) and V/N titers. However, the PEDV N protein ELISA is a reliable tool to distinguish between and select positive and negative samples for assay validation. In addition to the ELISA, samples were also screened by an indirect immunofluorescence assay (IFA). Only samples with similar results on the ELISA and IFA were selected for testing in this study. As expected no cross-neutralization was detected in the non-specific control sera tested. Antigenic cross-reactivity, but not cross-neutralization, of PEDV and other related swine coronaviruses such as the transmissible gastro-enteritis virus (TGEV), porcine respiratory and enteric coronavirus (PRCV) and porcine delta-coronavirus (PDCoV) has been previously demonstrated (Lin et al., 2015).

Therefore, the performance of the newly developed colorimetric assays was comparable to the conventional assays for the quantification of PEDV virus cultures and neutralizing antibodies. The availability of an OD cut-off value to distinguish between wells with and without CPE can eliminate visual plate reading by operators to significantly improve the turnaround time in diagnostic laboratories with a high volume of testing for PEDV virus isolations and V/N tests.

Funding

This study was supported by the Agricultural Products Utilization Committee (APUC) of N. Dakota, the N. Dakota State Board of Agricultural Research (SBARE) and the United States Department of Agriculture (USDA) under project ND02425.

Conflict of interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Acknowledgements

The authors thank the staff of the NDSU Microbiological Sciences Department and the North Dakota State University Veterinary Diagnostic Laboratory for their technical assistance. We thank Ms. Heather Vinson for proof-reading the manuscript and Dr. Linda Saif for providing anti-sera.

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