ACTA IMEKO
ISSN: 2221-870X
September 2025, Volume 14, Number 3, 1 - 5

Are real-time and droplet-digital polymerase chain reaction qualitative results comparable in detecting Bovine Viral Diarrhea Virus ribonucleic acid?

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ABSTRACT
Using Polymerase Chain Reaction (PCR) or Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) to detect viral agents during the acute phase of infection is becoming increasingly common. To ensure the accuracy of these in vitro diagnostic tests, studies are ongoing to develop Reference Materials (RM) for molecular assays, using Bovine Viral Diarrhea Virus (BVDV) as a model. Qualitative methods for detecting BVDV ribonucleic acid (RNA) are employed to diagnose the disease in animals and to detect contamination in cell cultures. To establish a reliable routine for detection of BVDV RNA, a commercial Reverse Transcription quantitative PCR (RT-qPCR) was briefly compared to Reverse Transcriptase Droplet Digital PCR (RT-ddPCR, a primary method) using the same primers and probe. Using both methods, serial dilutions of BVDV RNA standard and BVDV RNA grown in cell culture were evaluated, with RT-qPCR showing 100 % sensitivity, with specificities of 83 % and 67 %, respectively. Discrepancies between the two techniques were observed at low RNA concentrations, with possible false positives in RT-qPCR. This finding highlights the importance of stringent validation in molecular in vitro diagnostic (IVD) methods.

Section: RESEARCH PAPER

Keywords: digital PCR; RT-qPCR; BVDV; RNA; IVD

Citation: A. M. Rodrigues, M. R. F. Henrique, J. L. P. Ramos-Jr, R. B. Flatschart, V. de Souza, J. M. Granjeiro, A. V. Folgueras-Flatschart , Are real-time and droplet-digital polymerase chain reaction qualitative results comparable in detecting Bovine Viral Diarrhea Virus ribonucleic acid?, Acta IMEKO, vol. 14 (2025) no. 3, pp. 1-5. DOI: 10.21014/actaimeko.v14i3.2012

Section Editor: Carlos Hall, PósMQI/PUC-Rio, Rio de Janeiro, Brazil

Received November 30, 2024; **In final form** September 2, 2025; **Published** September 2025

Funding: This work was supported by Capes, Faperj, Pronametro and Inmetro, Brazil.

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1. INTRODUCTION

Polymerase Chain Reaction (PCR) and Reverse Transcription PCR (RT-PCR) are increasingly common methods for detecting viral agents during the acute phase of infections by identifying their nucleic acids, Deoxyribonucleic Acid (DNA) or Ribonucleic Acid (RNA) in biological samples. These techniques gained widespread recognition during the Coronavirus Disease 2019 (COVID-19) pandemic, when RT-PCR was extensively used to detect Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) RNA, playing a key role in patient screening and disease control efforts [1].

To ensure the accuracy of these In Vitro Diagnostic (IVD) tests in laboratories accredited under International Organization for Standardization (ISO) 15189:2022 [2] or International Organization for Standardization / International Electrotechnical Commission (ISO/IEC) 17025:2017 [3], a feasibility study was conducted for producing Reference Materials (RM) to molecular assays, using Bovine Viral Diarrhea Virus (BVDV) as a model. BVDV, a pestivirus from the Flaviviridae family, is an enveloped virus with a 12.3 kb single-stranded, positive-sense RNA genome [4]. Due to its genetic similarity to the Hepatitis C Virus (HCV), BVDV is commonly used to validate viral inactivation methods and evaluate antiviral

therapies [5]. Moreover, BVDV could also serve as a model for the development of an RM for molecular assays of other Flaviviridae (such as those causing Yellow Fever, Dengue, or Zika) or RNA viruses like SARS-CoV-2.

Bovine Viral Diarrhea (BVD) is an infectious disease endemic in many countries, caused by BVDV, which is the primary contaminating virus in Fetal Bovine Serum (FBS). FBS is a supplement used in cell culture media to promote cell growth and is also a key ingredient in the production of biological products, including vaccines and biopharmaceuticals. Molecular diagnostic methods, such as Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) are commonly used to detect BVDV RNA and help control the disease in cattle, as well as assess contamination in cell cultures and in raw materials of biological origin.

To establish a molecular method for detecting BVDV RNA and to evaluate the homogeneity and stability of future RM batches, this study compares the performance of two techniques, RT-qPCR and Droplet Digital Reverse Transcription Polymerase Chain Reaction (RT-ddPCR), in detecting the virus in serial dilutions. While RT-ddPCR is regarded as the gold standard for absolute nucleic acid quantification (copies/µl) due to its high precision and sensitivity [6], [7], it can also be used in qualitative assessments.

2. METHODOLOGY

2.1. BVDV

Two types of materials containing BVDV RNA were used: (i) a standard positive control from the commercial VetMAX™ Gold BVDV PI Detection Kit (Thermo Fisher Scientific), and (ii) virus produced in culture, specifically the Singer strain of BVDV grown in Madin-Darby Bovine Kidney (MDBK) cells. Horse serum was used as a supplement in the culture medium.

2.2. RNA extraction from cell-cultured BVDV

RNA from the Singer strain was extracted in duplicate with the PureLink™ Viral RNA/DNA Mini Kit (Thermo Fisher Scientific) following the manufacturer's instructions (purelink_viral_rna_dna_man.pdf) in a final elution volume of 30 µL.

2.3. Standard dilutions

Six serial dilutions (in base 10) were prepared using the 25x BVDV Control RNA standard from the VetMAX-Gold BVDV PI Detection Kit (Thermo Fisher Scientific). According to the manufacturer, the concentration of the 25x BVDV Control RNA is 10,000 copies/µL.

2.4. Dilutions of BVDV RNA from cell culture

Eight serial dilutions (in base 10) were prepared using RNA extracted from Singer strain infected cell cultures.

2.5. RT-qPCR

VetMAX™-Gold BVDV PI Detection Kit (valided by Thermo Fisher Scientific and USDA licensed) was employed to perform qualitative RT-qPCR assays, according to the manufacturer's instructions (4425603_BVDV_USDA_IFU.pdf), on the Applied Biosystems™ 7500 Real-Time PCR System (SDS v 2.3). Positive and negative controls and all samples were tested in duplicate. In each valid plate, samples with a Cycle Threshold (Ct) value $Ct < 38$ were considered positive, while those with no amplification were considered negative.

2.6. RT-ddPCR

The same primers and probe used in RT-qPCR (from the VetMAX™-Gold BVDV PI Detection Kit), with reagents from the One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad), were used for RT-ddPCR. The amplification conditions for RT-ddPCR were previously optimized [8], with Reverse Transcriptase (RT) temperature set at 50 °C and Annealing temperature (T_a) at 60 °C. After thermocycling, results were read on Droplet Reader QX200 (Bio-Rad) and analysed through QuantaSoft™ Software v 1.7.4. All dilutions were tested in duplicate. Samples with an RNA quantification assigned were considered positive, while those with no quantification shown were considered negative.

2.7. Comparison of RT-qPCR and RT-ddPCR Results

The results from RT-qPCR (expressed as *Ct* values), RT-ddPCR (copies/µL), and their qualitative interpretation (positive or negative) were presented in tables based on the duplicate results from each dilution. The sensitivity, specificity, and predictive values (with a 95 % confidence interval) of RT-qPCR relative to RT-ddPCR were assessed using the MedCalc Software Ltd. Diagnostic Test Evaluation Calculator (https://www.medcalc.org/calc/diagnostic_test.php, Version 23.0.9; accessed 29 August 2025).

2.8. Receiver Operating Characteristic (ROC) curves

ROC curves were constructed from the experimental values of the False Positive Rate (FPR) and True Positive Rate (TPR) obtained for each dilution condition. The Area Under the Curve (AUC) was estimated using the trapezoidal rule by linearly connecting adjacent points. This calculation was performed with the metrics.auc function from the scikit-learn library [9]. ROC curve visualization was carried out using the Matplotlib library [10], including the reference curve (diagonal line, $AUC = 0.5$), which represents random performance.

3. EXPERIMENTAL RESULTS WITH EVALUATION

The results obtained from the duplicates of the six dilutions of the 25x BVDV Control RNA, using both RT-qPCR and RT-ddPCR, are presented in Table 1, and the results from duplicates of the eight dilutions of BVDV RNA, extracted from MDBK cells infected with the Singer strain, using both RT-qPCR and RT-ddPCR, are presented in Table 2.

A comparison of the qualitative results from both techniques (RT-qPCR and RT-ddPCR) for the 25x BVDV Control RNA dilutions is shown in Table 3. The sensitivity value of RT-qPCR was 100 %, while its specificity value relative to RT-ddPCR (considered the reference method) was 83.3 %. Results obtained from the dilutions of BVDV RNA extracted from culture are shown in Table 4. The sensitivity value of RT-qPCR was 100 %, while its specificity value relative to RT-ddPCR (considered the reference method) was 66.7 %.

Discrepancies were observed when comparing the qualitative results assigned to the samples by the two techniques. RT-qPCR presented positive results in some samples that were not amplified on RT-ddPCR. These discrepancies were apparent in highly diluted samples with low analyte concentrations (or even in the absence of the target). Given the results, potential false positives by RT-qPCR could be due to: (i) the higher sensitivity of the method compared to the reference method, (ii) the lower specificity of RT-qPCR (which may detect some interferents as being the analyte), or (iii) the presence of artifacts in the RT-

Table 1. RT-ddPCR and RT-qPCR results of six serial 10-fold dilutions of the 25x BVDV Control RNA standard (Thermo Fisher Scientific).

	RT-ddPCR		RT-qPCR	
Sample	*RNA* (copies/µl)	RT-ddPCR Qualitative result	*Ct*	RT-qPCR Qualitative result
Dil 1	4320	+	27.51	+
	4320	+	27.72	+
Dil 2	386	+	31.16	+
	388	+	31.22	+
Dil 3	9	+	36.32	+
	13.4	+	35.63	+
Dil 4	No Call	-	not determined	-
	No Call	-	37	+
Dil 5	No Call	-	not determined	-
	No Call	-	not determined	-
Dil 6	No Call	-	not determined	-
	No Call	-	not determined	-
NTC	No Call	-	not determined	-
	No Call	-	not determined	-

Six 10-fold serial dilutions (Dil. 1 to 6) of the 25x BVDV Control RNA standard (Thermo Fisher Scientific) were evaluated in duplicate. The negative control NTC (No Template Control) was also tested in duplicate. Samples with RNA quantification by RT-ddPCR or those with *Ct* < 38 on RT-qPCR were considered positive (+). Samples with no evidence of amplification (samples with "No Call" by RT-ddPCR or "not determined" by RT-qPCR) were considered negative (-).

Table 2. RT-ddPCR and RT-qPCR results of eight serial 10-fold dilutions of BVDV RNA extracted from infected cell culture.

	RT-ddPCR		RT-qPCR	
Sample	*RNA* (copies/µl)	RT-ddPCR Qualitative result	*Ct*	RT-qPCR Qualitative result
Dil 1	24000	+	18.83	+
	21250	+	18.83	+
Dil 2	3557.5	+	22.77	+
	3307.5	+	22.77	+
Dil 3	259.75	+	26.75	+
	237.5	+	26.75	+
Dil 4	16.25	+	30.81	+
	17.5	+	30.81	+
Dil 5	1.75	+	34.80	+
	1.4	+	34.30	+
Dil 6	No Call	-	not determined	-
	No Call	-	36.97	+
Dil 7	No Call	-	not determined	-
	No Call	-	36.95	+
Dil 8	No Call	-	not determined	-
	No Call	-	not determined	-
NTC	No Call	-	not determined	-
	No Call	-	not determined	-

Eight serial 10-fold dilutions (Dil. 1 to 8) of Singer strain BVDV RNA produced in MDBK cells were evaluated in duplicate. The negative control NTC (No Template Control) was also tested in duplicate. Samples with RNA quantification by RT-ddPCR or those with *Ct* < 38 on RT-qPCR, were considered positive (+). Samples with no evidence of amplification (samples with "No Call" by RT-ddPCR or "not determined" by RT-qPCR) were considered negative (-).

qPCR reaction, generating fluorescence at higher *Ct* values (e.g., ≥ 38).

In this comparison of the two techniques, the same primers and probe from the RT-qPCR kit (validated by the manufacturer) were used in both assays, which suggests that differences in specificity between the techniques are unlikely. Additionally, since reaction conditions are not interchangeable between the two methods [11], [12], [13], the RT and annealing temperatures for RT-ddPCR were optimized. This adjustment enhances the sensitivity of RT-ddPCR, which is typically reported to be equal to or greater than that of RT-qPCR [7], [14].

Given these factors, determining the exact cause of the false positives was challenging, as the VetMAX™-Gold BVDV PI Detection Kit does not provide details on the reagent

Table 3. Sensitivity and specificity of the RT-qPCR compared to RT-ddPCR, both used as qualitative methods for detecting BVDV RNA in serially diluted 25x BVDV Control RNA standard.

	RT-ddPCR	
RT-qPCR	+	-
+	6	1
-	0	5

Statistic	Value	95 % CI
Sensitivity	100.00 %	54.07 % to 100.00 %
Specificity	83.33 %	35.88 % to 99.58 %
Positive Likelihood Ratio	6.00	1.00 to 35.91
Negative Likelihood Ratio	0.00	-
Positive Ratio (*)	50.00 %	21.09 % to 78.91 %
Positive Predictive Value (*)	85.71 %	50.06 % to 97.29 %
Negative Predictive Value (*)	100.00 %	47.82 % to 100.00 %
Accuracy (*)	91.67 %	61.52 % to 99.79 %

(*) values are dependent on a positive ratio.

Comparative analysis of positive (+) and negative (-) results in both techniques: RT-qPCR and RT-ddPCR. Analysis with a 95 % confidence interval (CI) was adapted from MedCalc Software results, Diagnostic Test Evaluation Calculator, https://www.medcalc.org/calc/diagnostic_test.php (Version 23.0.9; accessed 29 August 2025).

Table 4. Sensitivity and specificity of RT-qPCR compared to RT-ddPCR for qualitative detection of BVDV RNA extracted from cell culture.

	RT-ddPCR	
RT-qPCR	+	-
+	10	2
-	0	4

Statistic	Value	95 % CI
Sensitivity	100.00 %	69.15 % to 100.00 %
Specificity	66.67 %	22.28 % to 95.67 %
Positive Likelihood Ratio	3.00	0.97 to 9.30
Negative Likelihood Ratio	0.00	-
Positive Ratio (*)	62.50 %	35.43 % to 84.80 %
Positive Predictive Value (*)	83.33 %	61.72 % to 93.94 %
Negative Predictive Value (*)	100.00 %	39.76 % to 100.00 %
Accuracy (*)	87.50 %	61.65 % to 98.45 %

(*) values are dependent on a positive ratio.

Comparative analysis of positive (+) and negative (-) results in both techniques: RT-qPCR and RT-ddPCR. Analysis with a 95% confidence interval (CI) was adapted from MedCalc Software results, Diagnostic Test Evaluation Calculator, https://www.medcalc.org/calc/diagnostic_test.php (Version 23.0.9; accessed 29 August 2025).

composition, primers and probe sequences, or on the validation process used to establish the cutoff for positive results (e.g., *Ct* < 38). Therefore, to perform a feasibility study for an RM, it was deemed essential to validate a fully controlled method. This validation process is also a key responsibility for kit developers and manufacturers, who determine the method's characteristics (such as sensitivity and specificity) by comparing the results with an external reference, such as an RM. The availability of RM remains crucial for testing laboratories to verify and maintain the quality of diagnostic methods [15].

Validating molecular methods for detecting or quantifying viral RNA or DNA is a critical issue, with significant implications for disease diagnostics and control [1]. A recent example highlights the need to reconsider the validation of qualitative molecular methods for viral infections. During the COVID-19 pandemic, RT-qPCR was the gold standard for detecting SARS-CoV-2, with a cutoff of *Ct* < 38 for positive samples. However, as the understanding of the method and the disease improved, the cutoff was revised to *Ct* < 28, with samples showing a *Ct* ≥ 28 considered a possible indication of nonspecific amplification [16].

When evaluating the performance of RT-qPCR in comparison with RT-ddPCR using dilutions of the 25x BVDV Control RNA standard (Thermo Fisher Scientific), a Receiver Operating Characteristic (ROC) curve (Figure 1) was constructed. In Dilutions 1 to 3 (Dil 1–3), the performance of RT-qPCR was optimal, with all positive samples correctly identified. These points are clustered in the upper-left corner of the ROC curve, which represents the optimal scenario. At Dilution 4 (Dil 4), however, performance declined, falling below the random line, indicating worse-than-random classification. In Dilutions 5 to 6 (Dil 5–6), and in the NTC (significantly diluted or intentionally absent analyte), no positive samples were available for detection, making the classification of true positives impossible. In these latter dilutions and in the NTC, true negatives were correctly classified, with no false positives observed, and thus recorded as 0:0.

When considering all dilutions simultaneously, the test does not discriminate well between positives and negatives. However, when restricting the analysis to Dilutions 1 to 5 (Dil 1–5), performance is excellent (with Area Under the Curve [AUC] equal to 1).

Still comparing RT-qPCR against RT-ddPCR using dilutions of BVDV RNA extracted from infected cell cultures, the ROC curve (Figure 2) showed a pattern similar to the one obtained previously, with the test performing very well in Dilutions 1 to 5 (Dil 1–5) and completely losing its discriminative power at higher dilutions (Dil 6 onward and NTC).

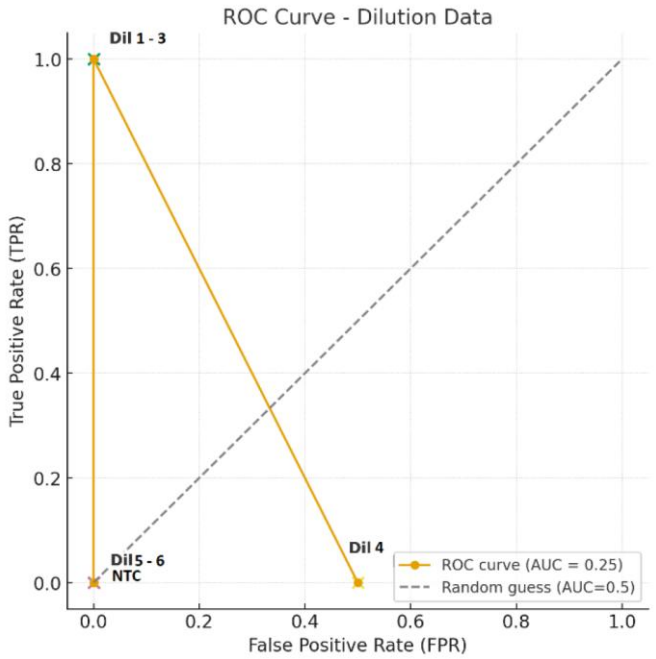

Figure 1. Receiver Operating Characteristic (ROC) curve with Area Under the Curve (AUC) and the random reference line for six serial 10-fold dilutions (Dil 1–6) of 25x BVDV Control RNA standard. The RT-qPCR method is evaluated against the RT-ddPCR method. The random line is dashed.

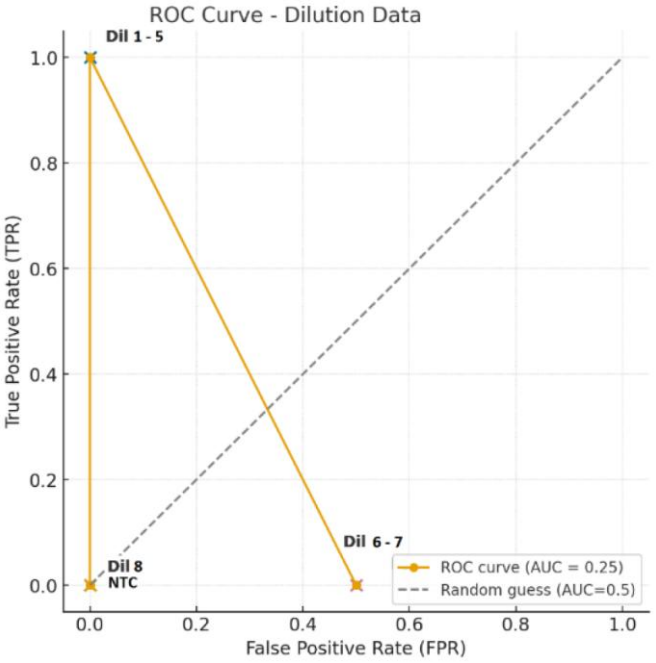

Figure 2. Receiver Operating Characteristic (ROC) curve with Area Under the Curve (AUC) and the random reference line for eight serial 10-fold dilutions (Dil 1–8) of BVDV RNA from cell culture. The RT-qPCR method is evaluated against the RT-ddPCR method. The random line is dashed.

4. CONCLUSIONS

This study evaluated the use of a commercially validated RT-qPCR kit in comparison with an optimized RT-ddPCR method (using the same set of primers and probe) for detecting BVDV RNA, with the aim of its application in routine laboratory workflows for developing Reference Materials. When comparing qualitative results, discrepancies were observed among replicates at dilutions with low RNA concentrations. It is well known that fluctuations in analyte quantification are common when measurements are performed near the detection limits of the methods. In the context of in vitro diagnostic methods, such as the RT-qPCR assay evaluated here, which support disease management and control actions, the occurrence of false positives is often a deliberate choice made by kit developers, as a consequence of the high sensitivity sought. However, for applications as a laboratory tool in processes such as the evaluation and monitoring of the production of standards or qualitative Reference Materials, the occurrence of false positives may have essential implications for homogeneity, stability, and characterization studies.

Additional challenges arose when attempting to adapt commercial quantitative PCR (qPCR) kits – or their primers and probes – for the optimization of digital PCR (dPCR) assays for the intended purpose. The use of ready-made primer/probe mixes and the unavailability of their sequences prevented even minimal evaluations (in silico or in vitro) of potential dimer formation or nonspecific amplification. The lack of detailed information regarding kit components and method validation also made it challenging to perform in-depth analyses when questions or potential issues emerged.

These findings underscore the need for the development, optimization, and validation of RT-ddPCR methods tailored to tasks related to the production and characterization of standards or reference materials. Accordingly, we are currently validating a method for the absolute quantification of BVDV RNA in samples of fetal bovine serum or cell culture matrices. This effort may contribute to advancing knowledge and developing metrological tools (such as RM) that ensure the accuracy and reliability of molecular test results in virology.

AUTHORS' CONTRIBUTION

The initial idea for the work and the experimental design were conceived by Roberto B. Flatschart and Áurea V. Folgueras-Flatschart, who also supervised all activities. Ariane M. Rodrigues, Mayane R. F. Henrique, and José L. P. Ramos-Jr curated and formally analysed the experimental data. The manuscript was initially written by Ariane M. Rodrigues, Mayane R. F. Henrique, and Áurea V. Folgueras-Flatschart. Roberto B. Flatschart reviewed it. José M. Granjeiro and Vanderlea de Souza raised funds and managed the project.

ACKNOWLEDGEMENT

We thank Dr. Marcos Bryan Heinemann (USP-SP, Brazil) for generously providing the Singer strain of BVDV and Capes, Faperj, Pronametro, and Inmetro for financial support.

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