Comparison of RT-qPCR and RT-ddPCR methodologies for the detection of Bovine Viral Diarrhea Virus (BVDV) RNA

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Abstract. The Bovine Viral Diarrhea Virus is responsible for causing infection of great importance in cattle, being highly disseminated in the world and endemic in Brazil. This virus is also described as the main contaminating virus of Fetal Bovine Serum (FBS), a supplement routinely used in cell cultures and which can become a source of contamination for these cells. Qualitative methods for detecting BVDV RNA can be used both for the diagnosis of the disease in animals and to detect the infection of cultures. In this study, we compared the results of a commercial diagnostic method by Real Time RT-PCR (RT-qPCR) to the RT-Droplet Digital PCR (RT-ddPCR) method using the same set of probe and primers. Serial dilutions of the BVDV RNA standard from the commercial kit and also the BVDV RNA produced in MDBK cells were evaluated by both methods. RT-qPCR showed 100% sensitivity and 80% to 83% specificity compared to the RT-ddPCR method. Points of disagreement between the two techniques arose at low RNA concentrations.

1. Introduction

Bovine Viral Diarrhea is an endemic infectious disease in many countries, caused by Bovine Viral Diarrhea Virus (BVDV), a Pestivirus of the *Flaviviridae* family. It has single-stranded RNA with positive polarity, is enveloped and its genome is 12.3 kb in size [1]. BVDV is described as the main contaminating virus of Fetal Bovine Serum (FBS), a supplement used in culture media that has the ability to nourish and stimulate cell growth in vitro, being considered a raw material for the production of biological products such as vaccines and biopharmaceuticals [2]. Both for controlling the disease in cattle and for evaluating the contamination of cell cultures, molecular diagnostic methods for direct detection of BVDV RNA can be used, as the traditional use of Real Time RT-PCR (RT-qPCR). The RT-Droplet Digital PCR (RT-ddPCR) method, more recent and considered a reference method for the absolute quantification of nucleic acids in copies/µl [3], with high precision and sensitivity [4], can also be used for qualitative tests.

In the present study, we compared the performance of the two techniques, RT-qPCR and RT-ddPCR, regarding their ability to detect BVDV RNA in serial dilutions of the analyte. The study was

motivated by previous observation of discrepant results using the two techniques for FBS samples analysis.

2. Methodology

2.1. BVDV

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

2.2 RNA Extraction from BVDV produced in cell culture

RNA extraction from Singer strain was performed in duplicate using the PureLink™ Viral RNA/DNA Mini Kit (according to manufacturer's instructions, Thermo Fisher Scientific) and a final RNA elution volume of 30µL.

2.3. Serial dilutions

2.3.1. BVDV Control RNA dilutions

Using the standard 25x BVDV Control RNA from the VetMAX-Gold BVDV PI Detection Kit (Thermo Fisher Scientific), 6 serial 10-fold dilutions were prepared. According to the manufacturer, the concentration of the 25x BVDV Control RNA is 10,000 copies/µl.

2.3.2. Cell culture derived BVDV RNA dilutions

Singer strain RNA extracted from MDBK culture was used in the preparation of 8 serial 10-fold dilutions.

2.4. RT-qPCR

In carrying out the qualitative tests by RT-qPCR, the VetMAX™-Gold BVDV PI Detection Kit (Thermo Fisher Scientific) was used, following the manufacturer's guidelines, and the 7500 Real Time PCR System from Applied Biosystems™, SDS v 2.3. All samples were tested in duplicate. Samples with results with Ct<38 were considered positive. Those with Ct ≥38 or absence of amplification were considered negative.

2.5. RT-ddPCR

In carrying out the RT-ddPCR, we utilized the same probe and primers used in the RT-qPCR (from the VetMAX™-Gold BVDV PI Detection Kit), the reaction reagents from the One – Step RT-ddPCR Advanced kit for Probes (Bio- Rad) and the C1000 Touch Thermal Cycler (Bio-Rad). The amplification program, otimized for RT-ddPCR, was the same used in RT-qPCR. After thermocycling, the results were read using a QX200 Droplet Reader (Bio-Rad) and analyzed using QuantaSoft™ Software v 1.7.4. All samples were tested in duplicate. Samples that showed RNA quantification were considered positive and those without quantification being negative.

2.6. RT-qPCR and RT-ddPCR results comparision

The comparison of the results obtained by RT-qPCR (Ct), by RT-ddPCR (copies/µL) and the interpretation of the qualitative results (positive and negative) were performed in tables and histograms constructed from the results obtained for each dilution. The evaluation of sensitivity and specificity of RT-qPCR in relation to RT-ddPCR was performed using the MedCalc software (https://www.medcalc.org/calc/diagnostic_test.php).

3. Results and Discussion

3.1. Results comparison of 25x BVDV Control RNA dilutions

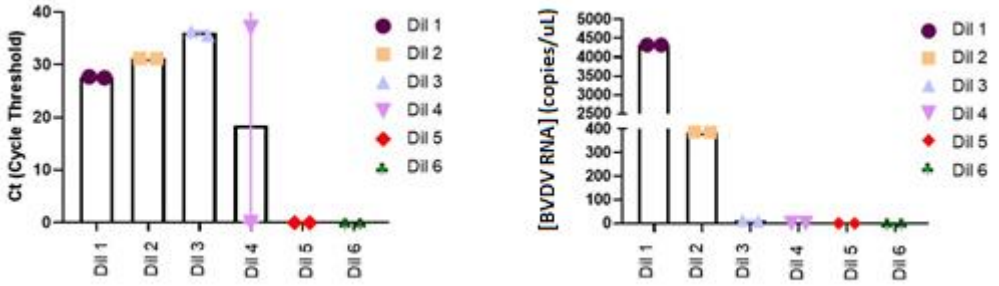
The RT-qPCR and RT-ddPCR results obtained for duplicates from 6 dilutions of 25x BVDV Control RNA are presented in Table 1 and, graphically, in Figure 1.

Table 1: RT-ddPCR and RT-qPCR results of the 6 10-fold dilutions of the 25x BVDV Control RNA standard (Thermo Fisher Scientific).

		RT-ddPCR		RT-qPCR	
Sample	Expected [RNA] (copies/µl)	Observed [RNA] (copies/µl)	Qualitative result	Ct	Qualitative result
Dil 1	1000	4320	+	27,51	+
		4320	+	27,72	+
Dil 2	100	386	+	31,16	+
		388	+	31,22	+
Dil 3	10	9	+	36,32	+
		13,4	+	35,63	+
Dil 4	1	No Call	-	not determined	-
		No Call	-	37	+
Dil 5	0,1	No Call	-	not determined	-
		No Call	-	not determined	-
Dil 6	0,01	No Call	-	not determined	-
		No Call	-	not determined	-
NTC	0	No Call	-	not determined	-
NTC		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 evaluated in duplicate. Samples with RNA quantification at RT-ddPCR, as well as those with Ct<38 at RT-qPCR, were considered positive (+). Samples with no evidence of amplification (samples as "No Call" by RT-ddPCR or "not determined" by RT-qPCR) were considered negative (-).

Figure 1: Results of 25x BVDV Control RNA dilutions using RT-qPCR and RT-ddPCR methods.



The histogram on the left shows the RT-qPCR results, with the Ct value on the Y axis. The histogram on the right shows the RT-ddPCR results, with the observed RNA concentration on the Y axis (copies/µl for each dilution). In both histograms, on the X axis, the 6 serial 10-fold dilutions of the BVDV control RNA are indicated. Each of the duplicates is represented individually for each dilution.

Results comparison of RT-qPCR and RT-ddPCR qualitative assays applied to 25x BVDV Control RNA standard dilutions are shown in Table 2. It was possible to calculate a sensitivity of 100% and specificity of 83% for the RT-qCR technique compared to RT-ddPCR.

Table 2: Sensitivity and specificity of the RT-qPCR technique in relation to RT-ddPCR, both used as qualitative methods in the detection of BVDV RNA in serial dilutions of the standard 25x BVDV Control RNA.

	RT-ddPCR		
RT-qPCR	+	-	True Positive = 6 False Positive = 1
+	6	1	True Negative = 5
-	0	5	False Negative = 0 Sensitivity = 100% Specificity = 83%

Comparison of the number of positive (+) and negative (-) results in both techniques, RT-qPCR and RT-ddPCR. Sensitivity and specificity analysis by MedCalc software (https://www.medcalc.org/calc/diagnostic_test.php).

3.2. Results comparisions of cell extracted BVDV RNA dilutions

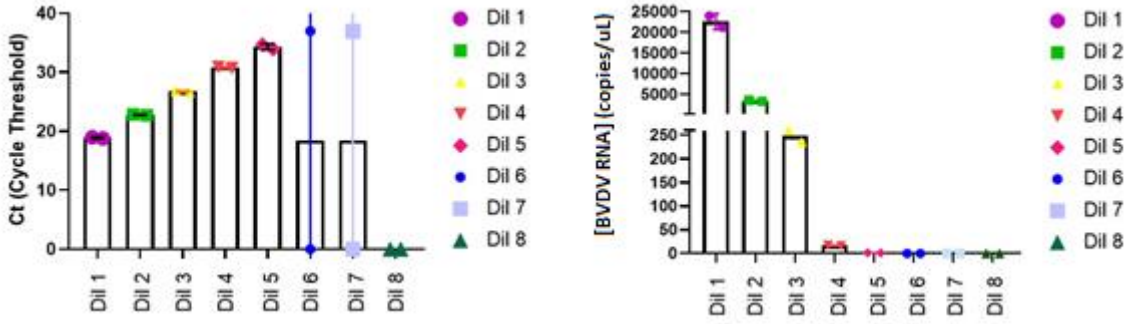
The RT-qPCR and RT-ddPCR results obtained for duplicates from 8 dilutions of BVDV RNA (extracted from cultured MDBK cells infected by the Singer strain) are presented in Table 3 and, graphically, in Figure 2.

Table 3: RT-ddPCR and RT-qPCR results of 8 serial 10-fold dilutions of BVDV RNA extracted from infected cell culture.

	RT-ddPCR		RT-qPCR	
Sample	[RNA] (copies/µl)	Qualitative result	Ct	Qualitative result
Dil 1	24.000	+	18,83	+
	21.250	+	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	-
	0,275	+	36,95	+
Dil 8	No Call	-	not determined	-
	No Call	-	not determined	-
NTC	No Call	-	not determined	-
NTC	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 evaluated in duplicate. Samples with RNA quantification at RT-ddPCR, as well as those with Ct<38 at RT-qPCR, were considered positive (+). Samples with no evidence of amplification (samples as “No Call” by RT-ddPCR or “not determined” by RT-qPCR) were considered negative (-).

Figure 2: Results of BVDV RNA dilutions extracted from culture using RT-qPCR and RT-ddPCR methods.



The histogram on the left shows the RT-qPCR results, with the Ct value on the Y axis. The histogram on the right shows the RT-ddPCR results, with the observed RNA concentration on the Y axis (copies/µl in each dilution). In both histograms, on the X-axis, the 8 serial 10-fold dilutions of the BVDV RNA extracted from cultured MDBK cells infected with the Singer strain are indicated. Each of the duplicates is represented individually at the dilutions shown.

Results comparison of RT-qPCR and RT-ddPCR qualitative assays applied to culture-extracted BVDV RNA dilutions are shown in Table 4. It was possible to calculate a sensitivity of 100% and specificity of 80% by the RT-qPCR technique compared to RT-ddPCR.

Table 4: Sensitivity and specificity of the RT-qPCR technique in relation to RT-ddPCR, both used as qualitative methods in the detection of BVDV RNA extracted from cell culture.

	RT-ddPCR		
RT-qPCR	+	-	True Positive = 11 False Positive = 1
+	11	1	True Negative = 4
-	0	4	False Negative = 0 Sensitivity = 100% Specificity = 80%

Comparison of the number of positive (+) and negative (-) results in both techniques, RT-qPCR and RT-ddPCR. Sensitivity and specificity analysis by MedCalc software (https://www.medcalc.org/calc/diagnostic_test.php).

Currently, ddPCR is accepted internationally as a reference method for the quantification of nucleic acids by the Bureau International des Poids et Measures (BIPM), the Joint Committee for Traceability in Laboratory Medicine (JCTLM) [5] and several National Institutes of Metrology (INM) [6, 7]. Using this technique to detect BVDV RNA and a commercical RT-qPCR, we observed the occurrence of some contradictions, with positive results by RT-qPCR, in some samples that were not amplified in RT-ddPCR, observed in dilutions with very low concentration of the analyte or in its absence. To confirm the cause of these results observed, additional experiments would be necessary. However, we can raise some hypotheses such as: (i) the low sensitivity of the RT-ddPCR procedure used or (ii) the occurrence of artifacts in the RT-qPCR procedure, presenting false-positive results.

While previously published works describe ddPCR as a more sensitive method when compared to qPCR [4], others alert us to the fact that the reaction conditions must be carefully established and that are not interchangeable between the two techniques. They highligh the importance of following steps:

choosing targets and oligonucleotide sequences, optimizing anealing and reverse transcription and method validation [8, 9, 10]. Then, by judicious carrying out these steps, background signals can be eliminated, allowing the detection and quantification of low-level target signals and improving the sensitivity of the method. For the best performance of an RT-ddPCR method, it is also worth considering the need to optimize the analyzes of raw ddPCR data, which can also contribute to reducing the number of false-positive droplets when the target is present in low copy number [9].

In this quick comparison of the two techniques, the optimization of the reaction conditions presented by the manufacturer in the RT-qPCR kit manual was considered and optimization of anealing and reverse transcription of the same reagents applied to RT-ddPCR was carried out. With the discrepancy in results between the two techniques, we had difficulty in judging the real probability of false positives occurring since the validation process that determined the cutoff at Ct38 (point that separates positive and negative results in RT-qPCR) is unknown to us, as is the composition of the reagents and the sequences of primers and probe(s).

Validating process of molecular methods for the detection or quantification of viral RNA or DNA is extremely important for the areas of human or animal health, with clear repercussions on the diagnosis and treatment of illnesses. A very recent example drew our attention to the need to review the validation of qualitative molecular methods applied to the diagnosis of viral infections. During the Covid-19 pandemic, RT-qPCR was used as the gold standard molecular method for detecting SARS-CoV-2 and, for a long time, the cutoff point for positive samples (with detectable amplification of viral RNA) was Ct<38 is also considered. However, with a better understanding of the method and the disease, the cutoff point was lowered to Ct 28, with samples with a Ct value<28 considered positive and a Ct value≥28 considered as a possibility of non-specific amplification [11]. Doubts about the sensitivity of the molecular diagnosis of Covid-19 led researchers to evaluate the use of ddPCR for this diagnosis as it detects low abundance targets. The work showed that RT-ddPCR increased the number of positive results by 8.6%, showing amplification in samples early considered negative or indetermined by RT-qPCR [12].

4. Conclusions

Analysis of sensitivity and specificity of RT-qPCR in relation to RT-ddPCR, using dilutions of the 25x BVDV Control RNA standard, showed sensitivity of 100% and specificity of 83%. The same analysis, when using dilutions of BVDV RNA extracted from culture, showed sensitivity of 100% and specificity of 80%.

Disagreements between the results found by the two techniques may be due to false positive results in RT-qPCR or lower sensitivity of RT-ddPCR. This discordance of results between replicates was observed in dilutions with low concentration of the RNA of interest. Fluctuations in quantification are common at low concentrations, when working close to the detection limit of the techniques.

Present data drew our attention to the need for a careful validation of molecular tests for the diagnosis of BVDV infection. For this, the availability of metrological tools, such as Reference Materials (MR) based on viral RNA will be important, supporting the efforts made by Inmetro to enable their production.

5. Aknolegment

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6. References

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