Comparison of the Clinical Validity of Droplet Digital PCR to ARMS-PCR for BRAF V600E Mutation Detection in Thyroid Nodules

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Funding information
This study was supported by Pearl River S&T Nova Program of Guangzhou (201710010178 and 201906010075), National Natural Science Foundation of China (81802551), 2018 Foshan Science and Technology SME Technology Innovation Project (F50AA-KJ019-4801-0015), and the Medical Research Fund from Guangzhou Sixth Affiliated Hospital (20190206).

Abstract
Objectives: Droplet digital PCR (ddPCR) has been reported to have a superior validity over PCR with amplification-refractory mutation system (ARMS-PCR) for detecting the BRAF V600E mutation in thyroid nodule fine-needle aspiration (FNA) samples using cytological diagnosis as the reference. However, the added value of ddPCR on surgical decision-making remains to be illustrated when the technique is combined with FNA cytology.

Methods: A total of 277 consecutive patients with thyroid nodules were subjected to FNA cytology and BRAF V600E testing with ARMS-PCR. Within this patient cohort, 90 patients underwent surgical intervention with pathological diagnosis available. BRAF V600E testing with ddPCR was performed retrospectively using FNA frozen DNA specimens. The clinical validity and utility of ddPCR in comparison with ARMS-PCR were compared using surgical pathology as the reference.

Results: Overall, 101 BRAF V600E mutations were detected by ddPCR, including five ARMS negative patients, four of whom were confirmed to have papillary thyroid cancer (PTC) by surgical pathology. Of the 90 patients with surgical pathology, which is considered the gold standard, ddPCR BRAF V600E testing yielded a sensitivity of 91.3% and specificity of 100% for PTC diagnosis, higher than that of ARMS (sensitivity 83.1%, specificity 100%). However, ddPCR only identified one more candidate patient for surgical intervention than ARMS when the techniques were combined with cytology.

Conclusions: This study highlighted the superior performance of ddPCR over ARMS in BRAF V600E detection from thyroid nodule FNA samples. Further studies are needed to evaluate the cost-effectiveness of replacing ARMS-PCR with ddPCR for surgical decision-making.

Keywords
BRAF V600E, droplet digital PCR (ddPCR), fine-needle aspiration (FNA), surgical decision-making, thyroid nodules
1 | INTRODUCTION

Thyroid nodules are common, with an incidence rate as high as 50%–70% in the adult population and are especially prevalent in women. The majority of thyroid nodules are benign, yet a small proportion become cancerous. Currently, ultrasound-guided fine-needle aspirate (FNA) cytology is the major method for the diagnosis of cancerous thyroid nodules. Unfortunately, the accuracy of FNA cytology remains unsatisfactory, with one-third of cases categorized as being diagnostic challenging. Therefore, many patients undertook unnecessary surgery or experienced a false-negative diagnosis due to inaccurate cytological testing.

Papillary thyroid cancer (PTC) accounts for over 80% of all thyroid cancers and has shown a dramatic increase in prevalence in recent years. The BRAF V600E mutation occurs in 50%–89% of PTC cases and serves as an important diagnostic and prognostic biomarker. The incorporation of BRAF V600E testing has been shown to substantially improve the diagnostic accuracy of FNA.

One of the most widely used methods for BRAF V600E detection in thyroid nodules is PCR with amplification-refractory mutation system (ARMS); this is a well-established technique that has been widely used for rapid detection of nucleic acid mutations in a wide variety of biological samples. However, ARMS-PCR may not be sensitive enough due to the fact that FNA samples usually have few mutant cells. Therefore, the development of a more sensitive and accurate detection method is warranted.

Droplet digital PCR (ddPCR) is a novel technology characterized by high sensitivity and absolute quantification of nucleic acid targets. The superior sensitivity renders ddPCR as a promising detection technique in samples with trace amounts of nucleic acids, such as in liquid biopsy. The Bio-rad QX200™ ddPCR was shown to improve diagnostic accuracy for detecting the BRAF V600E mutation by 17% compared to cytology alone in thyroid nodules FNA samples. A previous study has also reported the superior sensitivity of ddPCR over ARMS-PCR in PTC detection using the FNA cytology as the reference. However, as aforementioned, FNA cytological diagnosis is not accurate as surgical pathology, which is considered to be the gold standard; it remains unclear whether ddPCR has a better value than ARMS-PCR on surgical decision-making when in combination with FNA cytology. Therefore, in this study, we validated and compared the clinical utility of ddPCR to ARMS-PCR for the detection of the BRAF V600E mutation in FNA specimens of thyroid nodules, using pathological diagnosis following surgery as the gold standard.

2 | MATERIALS AND METHODS

2.1 | Study subjects and FNA samples collection

This is a retrospective cohort study involved consecutive patients who underwent FNA cytology and BRAF V600E testing with ARMS for thyroid nodules in The Guangzhou First People’s Hospital between October 2018 and July 2019. Based on the thyroid ultrasound findings, high-risk individuals were referred to ultrasound-guided FNA biopsy performed by trained physicians according to the recommended guideline. In addition to cytopathological examination, FNA specimens were also collected for BRAF V600E mutation testing. The pathologists who made the cytopathological diagnosis were blind to the ARMS-PCR and ddPCR results. The study was approved by the institutional ethical review board at The Guangzhou First People’s Hospital, and patients gave informed consent.

2.2 | Bethesda classification and surgical intervention

The Bethesda classification system is used for reporting FNA cytology. Based on this scheme, cases were divided into six categories: (I) nondiagnostic or unsatisfactory; (II) benign; (III) atypia of undetermined significance or follicular lesion of undetermined significance; (IV) follicular neoplasm or suspicious for a follicular neoplasm; (V) suspicious for malignancy; and (VI) malignant.

ARMS-PCR detection of BRAF V600E is the routine procedure for thyroid nodule FNA samples in the hospital. Patients with suspected cancer were referred to surgical intervention based on positive findings on ARMS BRAF V600E testing, FNA Bethesda category V/VI, and clinical consideration by the physicians. In total, surgery was performed in 90 cases and pathological diagnosis was used as the gold standard. The ddPCR test was performed retrospectively in September 2019 using the frozen FNA DNA samples; therefore, the ddPCR findings were not used for making treatment decisions in this study.

2.3 | Nucleic acid extraction from FNA samples

The Tissue DNA Extration Kit (AmoyDx, China) was used for DNA extraction from thyroid nodule FNA samples. After collection, FNA specimens were immediately transferred to a 1.5 mL microcentrifuge tube with 180 μL lysis buffer and DNA was extracted according to the manufacturer’s protocol. OD 260/280 was used to measure the DNA concentration.

2.4 | Plasmid preparation

The wild-type BRAF plasmid and the V600E mutant plasmid were first synthesized and then had their sequences confirmed by Generay Biotech Co., Ltd (Shanghai); Mutant and wild-type plasmids were prepared as the positive and negative controls. The QIAamp DNA mini kit (QIAGEN) was used for DNA extraction from plasmids. Qubit 4.0 (Thermo Fisher) was used to determine the concentration of plasmid DNA. Mutant plasmid DNA was diluted with wild-type plasmid DNA in a series of concentrations (0.05%, 0.1%, 0.5%, 1%,...
5%, 10%, and 50%) to determine the sensitivity of ddPCR for BRAF V600E detection.

2.5 | BRAF V600E mutation detection with ARMS-PCR and ddPCR

The ARMS-PCR assay was performed on an ABI 7500 Real-time PCR system (Life Technologist) with a BRAF V600E Diagnostic Kit (AmoyDx) according to the manufacturer's protocol. The details of this testing system have been published elsewhere.\(^{13}\)

The MicroDrop-100\(^{\text{TM}}\) ddPCR system (Forevergen), which is based on water-emulsion droplet technology, was used for the ddPCR assay. The primers and probes used for BRAF V600E detection with ddPCR are as follows:

- 5′-TGTTTTCTTACCTACTGAGCAGA-3′ (forward);
- 5′-CTAGCTACAGAAATC-MGB (mutant probe);
- VIC-CTAGCTACAGAAATC-MGB (wild-type probe).

The ddPCR assays were carried out in 20 \(\mu\)L reaction mixtures containing 10 \(\mu\)L ddPCR Supermix for Probes (Forevergen), 2 \(\mu\)L 10 \(\times\) BRAF V600E Mutant Assay (Forevergen), DNA templates, and deionized water. Droplets were generated using the MicroDrop-100\(^{\text{TM}}\) Droplet Generator (Forevergen) and then were moved into 96-well cartridges according to manufacturer's instructions. Amplifications were performed using the following conditions: 1 cycle of 95°C for 10 minutes, 45 cycles alternating between 95°C for 30 s and 60°C for 1 min, and 1 cycle of 98°C for 10 min before holding at 16°C. After amplification, the 96-well cartridges were placed into a MicroDrop-100B\(^{\text{TM}}\) detector (Forevergen) to measure the fluorescence signals. The mutation abundance for each sample was calculated using QuantDrop analysis software (Forevergen) following the principle of the Poisson distribution. The sample was regarded as a positive BRAF V600E mutation if there were three or more positive droplets.

The ARMS-PCR assay and the ddPCR assay were performed separately by two researchers to avoid mutual influences.

2.6 | Statistical analysis

Analysis was performed using the SPSS 20.0 software (IBM, USA). We used chi-square tests for comparisons between groups for categorical variables and two-tailed t tests for continuous variables. A \(P\) value < 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Cytological findings of FNA specimens

A total of 277 patients with both FNA cytology and ARMS-PCR BRAF V600E testing available were enrolled in this study. The mean age (±standard deviation) was 44.9 ± 13.2 years (range: 13-80), and 79.1% of the cohort (\(n = 219\)) was female. The cytopathology of FNA indicated that 86 cases (31.0%) were classified as malignant tumor (category VI) and 18 (6.5%) cases were suspicious for malignancy (category V), while 18 (6.5%) cases were classified as category III/IV with diagnostic challenge, and 43 (15.5%) cases were nondiagnostic (category I). The detailed description of the Bethesda classification is shown in Table 1.

3.2 | The sensitivity of BRAF V600E mutation testing with ddPCR

To evaluate the sensitivity of ddPCR, the BRAF V600E mutant plasmid was diluted with wild-type plasmids to V600E concentrations of 0.05%, 0.1%, 0.5%, 1%, 5%, 10%, and 50%. As shown in Figure 1, ddPCR was able to detect the BRAF V600E mutation at all dilutions and the calculated lowest copy number of the sample was approximately 1-2 copies/20 \(\mu\)L (0.05%). Additionally, the ddPCR results were highly reproducible in various BRAF V600E mutation dilutions: CV\(_{0.1\%}\) = 9.38%, CV\(_{1\%}\) = 5.81%, and CV\(_{10\%}\) = 0.99%.

3.3 | ddPCR vs. ARMS-PCR for BRAF V600E mutation detection

Using ARMS, we detected 96 (34.7%) cases with the BRAF V600E mutation, including 14 cases classified as Bethesda I-III, and 82 cases classified as Bethesda V-VI. Compared to ARMS, ddPCR detected five more mutant cases, of whom four cases were subjected to surgical intervention and PTC was confirmed by surgical pathological diagnosis, suggesting ddPCR has a higher sensitivity than ARMS on FNA BRAF V600E detection (Tables 2-3). Additionally, ddPCR detected the absolute quantity of BRAF V600E copies, which ranged from 6 to 5720 (0.05% to 43.4%) in the 20\(\mu\)L reaction system.

3.4 | FNA cytopathology, ARMS, and ddPCR vs. surgical pathology in a subgroup of patients

Of the 277 participants, 90 high-risk cases who had a positive finding on ARMS-PCR BRAF V600E and/or were classified as Bethesda category V/VI undertook surgical intervention. However, 35 high-risk cases with positive ARMS findings and/or Bethesda categories V/VI were lost to follow-up for surgical intervention. Nonetheless, the demographic and clinical characteristics were similar in the high-risk patients with or without surgical intervention (Table 1).

We compared the clinical validity of ARMS, ddPCR, and FNA cytopathology in this subgroup of patients using surgical pathology as the gold standard. As shown in Table 4, 83 out of 90 patients were diagnosed with PTC by pathological examination, including four cases diagnosed as benign (Bethesda II) by FNA cytopathology. In the subgroup of
patients categorized as Bethesda V/VI, FNA cytology showed 86.7% sensitivity and 71.4% specificity, with positive predictive value (PPV) of 97.3% and negative predictive value (NPV) of 25%. In contrast, all cases with the \( \text{BRAF} \) \( V600E \) mutation detected by ARMS and/or ddPCR were confirmed to have PTCs by pathological diagnosis, yielding a 100% specificity and 100% PPV for PTC diagnosis. Compared to ARMS, ddPCR alone had higher sensitivity and NPV. Additionally, when ddPCR was used to test patients categorized as Bethesda V/VI, the sensitivity reached up to 98.8% with 71.4% specificity, and only three cases were incorrectly diagnosed, confirming the superior clinical validity of ddPCR in \( \text{BRAF} \) \( V600E \) testing over ARMS.

### 3.5 The ddPCR \( \text{BRAF} \) \( V600E \) mutation rate by age, sonographic grade, and FNA cytology

To evaluate the association of the \( \text{BRAF} \) \( V600E \) mutation with age, we determined the V600E mutation rate by age group. Although...
older PTC patients were more likely to have the \textit{BRAF} V600E mutation, \textit{BRAF} V600E was more prevalent in younger individuals with thyroid nodules detected with ddPCR; both correlate significantly with aging (both $P$ for trend $<0.05$) (Figure 2A).

Unsurprisingly, \textit{BRAF} V600E mutations were more prevalent in individuals with higher TI-RADS and FNA Bethesda grades. Nevertheless, even in those with TI-RADS grade 3 or lower in the ultrasonic examination, there were still five (11%) patients with the \textit{BRAF} V600E mutation. Moreover, in patients categorized as Bethesda I, II, and III, the \textit{BRAF} V600E mutation rates were 12%, 4%, and 31%, respectively. These patients were most likely to experience false-negative diagnosis if only cytology was used, as was illustrated in the Venn diagram (Figure 2B-D).

4 | DISCUSSION

The \textit{BRAF} V600E mutation testing on FNA specimens from thyroid nodules greatly improves diagnostic accuracy.\textsuperscript{11,12} However, detecting trace amounts of DNA molecules in FNA specimens remains challenging. DdPCR is a cutting-edge technique that enables the sensitive and accurate detection of molecular markers from samples with a limited amount of target DNA.\textsuperscript{18} In this study, we confirmed the clinical validity of the MicroDrop-100\textsuperscript{TM} ddPCR system. It showed a better performance than ARMS-PCR in \textit{BRAF} V600E mutation detection on thyroid nodule FNA specimens, which is in line with a previous study.\textsuperscript{13} The combination of ddPCR and FNA cytology may serve as a better option for the diagnosis of thyroid nodules.

In this study, ddPCR detected five more mutant specimens than ARMS-PCR, three of which displayed mutation rates of less than 0.1%, illustrating the ultra-sensitivity of ddPCR. In support of this finding, four of these patients were confirmed to have PTC by pathological diagnosis following thyroidectomy. It is worth noting that one of these patients was classified as Bethesda category III; therefore, he or she might have been misdiagnosed if the decision was simply made according to FNA cytopathology and ARMS. Overall, our findings highlight the superior sensitivity of ddPCR over ARMS in \textit{BRAF} V600E detection in thyroid FNA specimens, especially when

\begin{table}[h]
\centering
\caption{Comparison of ARMS-PCR and ddPCR in \textit{BRAF} V600E detection in thyroid nodule fine-needle aspirate ($n = 277$)}
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{ARMS-PCR} & \textbf{ddPCR} & & & \\
\textbf{Negative} & \textbf{Positive} & \textbf{Total} & \textbf{P value} & \\
\hline
Negative & 176 & 5 & 181 & <.0001 \\
Positive & 0 & 96 & 96 & \\
Total & 176 & 101 & 277 & \\
\hline
\end{tabular}
\end{table}
only trace amounts of DNA molecules are in the sample, which is consistent with a previous report. However, it is also worth noting that ddPCR only identified one more candidate patient than ARMS when the technique was used in combination with Bethesda V/VI as the screening scheme for further surgical intervention. Thus, further studies with a larger sample size are still warranted to illustrate the cost-effectiveness of replacing ARMS with ddPCR as a companion test for FNA cytopathology.

The Bethesda classification system is the most widely used scheme for reporting FNA cytopathology. According to this scheme, surgical interventions are usually needed for patients with category V (suspicious for malignancy) and VI (malignant), due to the high probability for malignancy in these cases. Although only 6.5% of the cases in our study were classified as category III/IV, such cases are diagnostically challenging and diagnostic surgery may be required according to the 2017 Bethesda guideline; however, less than one-third of these cases were tested BRAF V600E positive in our study. Since BRAF V600E had 100% specificity for PTC according to the subgroup analysis using surgical pathology as the gold standard, two-thirds of these patients with category III/IV might have benign lesions and hence surgeries might not be necessary. Our findings are consistent with previous studies which also reported that less than one-third of patients with category III/IV were harboring cancer. Additionally, in this study, over half of thyroid nodules were classified in category I (undiagnosed) and II (benign) and the BRAF V600E mutation rates were 12% and 4%, respectively, with similar malignancy rates reported in previous studies. These malignant cases with category I/II might have been missed if diagnosis was simply based on FNA cytology. Given the huge number of patients with thyroid nodules, even a small difference in the misdiagnosis rate might translate into a tremendous burden on the healthcare system. Therefore, our data warrant the combination of BRAF V600E mutation testing to improve the diagnostic accuracy for the early detection of thyroid cancer.

The current study found a higher BRAF V600E mutation rate in younger people, with a significant decreasing trend over aging,
in line with the fact that PTCs are most likely to occur in young women. In the UK, it has been reported that the incidence rates of thyroid cancer in women reach a peak at ages 35-39 years and then decline steadily. In the United States, the analysis of the Surveillance, Epidemiology, and End Results-9 (SEER-9) cancer registry program revealed the highest thyroid cancer incidence rate at ages 40-59 years. Nevertheless, the current study identified the highest \textit{BRAF} \textit{V600E} mutation rate in ages around 30 years, suggesting an earlier onset age of thyroid cancer in Chinese women. Considering that young people constitute the majority of the workforce, the higher \textit{BRAF} \textit{V600E} mutation rate calls for an immediate initiative to develop more sensitive testing to improve early diagnosis. Additionally, over 80% of patients with PTCs were harboring the \textit{BRAF} \textit{V600E} mutation in this study, with a higher mutation rate in older patients, consistent with previous investigations. It remains unclear why older PTC patients tend to have a higher \textit{BRAF} \textit{V600E} mutation rate, although old people have a significantly lower risk for PTC than young people. We speculate that PTCs in young people might be more genetically heterogeneous and genetic risk predisposition other than the \textit{BRAF} \textit{V600E} mutation contributes to the earlier onset of thyroid carcinoma in these patients.

There are several major limitations in this study. Firstly, in our study, only high-risk individuals were referred to FNA biopsy and surgery; therefore, the diagnostic validity parameters, including sensitivity and specificity, were probably biased to a better level. Secondly, we did not perform post-operative testing to confirm the pre-operative \textit{BRAF} \textit{V600E} testing. Thirdly, it is worth noting that samples with low amounts of mutant copies still have a chance of a false-negative error. Lastly, this is a single center study with a limited sample size; therefore, the clinical utility of ddPCR in \textit{BRAF} \textit{V600E} testing should be confirmed in a larger population.

### Table 4

| Surgical pathology | Positive (n = 83) | Sensitivity | Specificity | PPV | NPV |
|--------------------|------------------|-------------|-------------|-----|-----|
| Bethesda classification | Negative (n = 7) | 3 | 3 | 86.7% | 71.4% | 97.3% | 25.0% |
|                     | Positive | 14 | 14 | 83.1% | 100% | 100% | 33.3% |
| ARMS-PCR | Negative | 0 | 69 | 83.1% | 100% | 100% | 33.3% |
|                     | Positive | 1 | 73 | 91.3% | 100% | 100% | 41.2% |
| Bethesda V/VI + ARMS-PCR | Negative | 5 | 1 | 97.6% | 71.4% | 97.6% | 71.4% |
|                     | Positive | 2 | 81 | 98.8% | 71.4% | 97.6% | 83.3% |
| Bethesda V/VI + ddPCR | Negative | 5 | 1 | 98.8% | 71.4% | 97.6% | 83.3% |
|                     | Positive | 2 | 82 | 98.8% | 71.4% | 97.6% | 83.3% |

Note: Bethesda classification: I, specimens nondiagnostic/unsatisfactory (ND/UNS); II, benign; III, atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS); IV, follicular neoplasm/suspicious for a follicular neoplasia (FN/SFN); V, suspicious for malignancy (SM); VI, malignancy.

Abbreviations: NPV, negative predictive value; PPV, positive predictive value.

### 5 | Conclusion

In spite of the limitations, this is the first study that compared the clinical validity of ddPCR to ARMS-PCR in \textit{BRAF} \textit{V600E} detection on FNA specimens from thyroid nodules using the pathological diagnosis following surgery as the gold standard. The ddPCR technique demonstrated superior performance over ARMS-PCR, particularly in trace amounts of target DNA specimens. However, it is also worth noting that compared to ARMS plus cytology, ddPCR plus cytology only identified one more candidate patient for surgical intervention. Therefore, further investigations with larger sample sizes and cost-effectiveness evaluation are needed to find out whether such slight superiority can be translated into substantial benefit to the healthcare system due to the extremely high prevalence of thyroid nodules.
ACKNOWLEDGMENT
We deeply acknowledge the contributions of physicians, nurses, and patients in this study.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
X.L, S.X, and Y.Z conceived the study and its design. X.L, H.D, and W.D were involved in the sample collection, reviewed, and edited the study. X.L, J.L, and J.H contributed to the testing, data analysis, and reviewed the study. X.L and Y.Z wrote the study. S.Z and Y.Z contributed to the discussion, and reviewed, edited, and finalized the study. All the authors read and approved the final study.

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How to cite this article: Li X, Du H, Luo J, et al. Comparison of the Clinical Validity of Droplet Digital PCR to ARMS-PCR for BRAF V600E Mutation Detection in Thyroid Nodules. J Clin Lab Anal. 2020;34:e23458. https://doi.org/10.1002/jcla.23458