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Diagnostic accuracy of a mutation-specific monoclonal antibody (RM8 clone) for BRAF V600E detection by immunohistochemistry in ameloblastomas

Abstract:

Objective: To determine the diagnostic accuracy of IHC using anti-BRAF V600E monoclonal antibody (RM8 clone) compared to Real-Time PCR in the detection of BRAF V600E mutation in FFPE ameloblastoma samples. **Methods:** This is a diagnostic accuracy study conducted based on the STARD recommendations. The index test was the IHC with anti-BRAF V600E antibody (RM8 clone), while the reference standard was the DNA analysis through castPCR™ mutation detection assays. The target condition was the detection of BRAF V600E mutation. The FFPE ameloblastoma samples were independently submitted to index test and reference standard. Sensitivity and specificity measures, and positive and negative predictive values were calculated. **Results:** Fifty-four FFPE ameloblastoma samples were included. The sensitivity and specificity of IHC using the RM8 clone compared to Real-Time PCR for the detection of the BRAF V600E mutation in FFPE ameloblastoma samples was 1.00 (95%CI 0.90–1.00) and 0.46 (95%CI 0.21–0.73), respectively, and the diagnostic test accuracy was calculated as 85.18%. **Conclusion:** IHC using BRAF V600E-specific antibody (RM8 clone) showed extremely high sensitivity, but suboptimal specificity when compared with Real-Time PCR in the detection of BRAF V600E mutation in FFPE ameloblastoma samples.

Keywords: Ameloblastoma; Immunohistochemistry; Mitogen-Activated Protein Kinase Kinases; Monoclonal Antibodies; Real-Time Polymerase Chain Reaction.

INTRODUCTION

The BRAF V600E mutation is a common activating mutation that has been linked to oncogenesis because of its disruptive impacts on cell signaling through the Mitogen-Activated Protein Kinase (MAPK) pathway^{1,2}. This molecular alteration was identified in about 65% of the ameloblastomas, which are a benign but locally aggressive odontogenic

tumor potentially eligible to targeted therapies with BRAF inhibitors³⁻⁵. Therefore, the evaluation of the

BRAF V600E mutation in these tumors could assume a crucial role in its management, emerging as a routine diagnostic procedure in the near future⁶.

Although the standard approach for detecting the BRAF V600E mutation in solid tumors

involves DNA analysis through molecular tests, These methods are costly and require complex infrastructure

Statement of Clinical Significance

The use of the RM8 clone for immunohistochemical detection of the BRAF V600E mutation in ameloblastomas shows high sensitivity but limited specificity, resulting in potential false positives. This impacts the diagnostic accuracy of this method, suggesting it is not yet suitable as a standalone diagnostic tool for clinical decision-making in routine practice.

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and specialized personnel⁷. The introduction of specific anti-BRAF V600E monoclonal antibodies has enabled its detection in formalin-fixed, paraffin-embedded (FFPE) tissue samples through immunohistochemistry (IHC), which is a more affordable alternative when compared to molecular tests^{6,8}.

Presently, two commercially available anti-BRAF V600E monoclonal antibodies are suitable for use in IHC: VE1 and RM8 clones. In contrast to the VE1 clone, a mouse monoclonal antibody produced with hybridoma technology, the RM8 clone is a recombinant monoclonal antibody generated through in vitro expression systems⁸. Although the exact immunogen sequences used to produce both the VE1 and RM8 clones remain proprietary information, they were designed to be BRAF V600E-specific, with no cross-reactivity with wild type BRAF.

Although IHC using the VE1 clone has shown good accuracy when compared to molecular tests⁹⁻¹³, studies on the performance of the RM8 clone are scarce and data on its sensitivity or specificity in ameloblastoma samples are not yet available. Therefore, the aim of this study was to determine the diagnostic accuracy of IHC using anti-BRAF V600E antibody (RM8 clone) compared to DNA analysis through Real-Time Polymerase Chain Reaction (PCR) in the detection of BRAF V600E mutation in ameloblastomas.

MATERIAL AND METHODS

Study design

This study was approved by the Ethics Committee of the University of Pernambuco (#4.309.512; CAAE: 35920620.7.0000.5192). This was a prospective diagnostic accuracy study, conducted according to the Standards for Reporting Diagnostic Accuracy (STARD) recommendations¹⁴. The index test was defined as the IHC using anti-BRAF V600E specific monoclonal antibody (RM8 clone), while the reference standard was the Competitive Allele-specific TaqMan™ Real-Time PCR Technology (castPCR™). The target condition was the detection of the BRAF V600E mutation.

Study population and sample size determination

The study population consisted of FFPE ameloblastoma specimens retrieved from the Pathology Department of a tertiary university hospital (Recife,

Pernambuco, Brazil). Histological sections, 5µm in thickness from each specimen, stained with hematoxylin and eosin, were examined by two researchers experienced in Oral and Maxillofacial Pathology to confirm the diagnosis. Tumors were classified according to WHO criteria³ and assessed for eligibility. Ameloblastomas in any clinicopathological were eligible for inclusion. Cases lacking sufficient or suitable biological material for IHC and molecular analyses were excluded.

The sample size calculation considered the expected prevalence of the BRAF V600E mutation in ameloblastomas (65%)⁴, estimated sensitivity and specificity for the index test (0.90 and 0.95, respectively, based on previous results⁶ obtained for the clone VE1), a maximum accepted margin of error of 10%, and a significance level of 95%. Consequently, the sample size required to determine diagnostic accuracy (sensitivity and specificity) was set at 54 cases.

Index test: immunohistochemistry with anti-BRAF V600E antibody (RM8 clone)

For immunohistochemical reactions, 3-µm-thick histological sections of each FFPE tumor sample were submitted to deparaffinization, rehydration and antigen retrieval using Trilogy solution (Cell Marque), followed by blocking of endogenous peroxidase and nonspecific binding. The primary anti-BRAF V600E specific antibody (RM8 clone, dilution 1:300. Thermo Fisher Applied Biosystems™) was incubated for 2 hours. Diaminobenzidine (DAB) was used as the chromogen for the reaction using HiDef HRP detection system (Cell Marque), and counterstaining was performed with Harris' hematoxylin. Positive (BRAF V600E ameloblastoma, previously confirmed by molecular tests) and negative (omission of the primary antibody) controls were carried out for each reaction.

The immunostaining in neoplastic cells was qualitatively and semi-quantitatively assessed by two independent examiners under a light microscope at magnifications corresponding to 100x and 400x. Ten random and representative fields were examined for each slide, and the estimated percentage of neoplastic cells with immunostaining was recorded. Reactions showing positive cytoplasmic staining in ≥10% of cells were classified as positive. Reactions with focal, scant (<10%), or absent staining were classified as negative, as previously recommended by Fregnani et al.¹⁵. Disagreements between the two examiners were resolved by consensus.

Reference standard: BRAF V600E detection by Real-Time polymerase chain reaction

From each case, 10µm sections of FFPE tumor tissue were processed using the MagMax™ FFPE DNA/RNA Ultra Kit (Applied Biosystems Thermo Fisher Scientific™), according to the manufacturer's instructions, to isolate genomic DNA. The double-stranded DNA concentration and purity were measured in Nano-Drop Lite spectrophotometer (Thermo Scientific™) and stored at -20°C until molecular analysis.

BRAF V600E mutation detection in DNA samples was performed in technical duplicates by Real-Time PCR using 40ng of template DNA combined with cast-PCR™ somatic mutation detection assays containing TaqMan™ probes specific for the c.1799T>A mutant *BRAF* allele (BRAF_476_mu) and for the reference *BRAF* gene (BRAF_rf) (Applied Biosystems Thermo Fisher Scientific™). The PCR amplification conditions followed the manufacturer's recommendations. Positive control (confirmed BRAF V600E template DNA) and No Template Control (NTC) were included in the reactions.

Amplification curves were imported to Mutation Detector™ software (Thermo Fisher Life Technologies), where the mutational status of the *BRAF* gene in each sample was analyzed. To ensure the blinding, the examiners assessed the results of the reference standard (Real-Time PCR) without knowledge of the results of the index test (IHC), and vice versa.

Additional molecular analysis: BRAF V600E detection by Sanger Sequencing

In instances of discordance between the index test and the reference standard, samples were submitted to Sanger Sequencing to confirm the mutational status. For this purpose, the exon 15 of *BRAF* gene was amplified in conventional PCR using DreamTaq Green PCR Master Mix (2X) (Applied Biosystems Thermo Fisher Scientific™) and the following primers: 5'-CTCTTCATA-ATGCTTGCTCTGATAGG-3' (forward) and 5'-AGTT-GAGACCTTCAATGACTTTCTAGT-3' (reverse). The amplification protocol consisted of initial denaturation at 95°C for 1 minute, 40 cycles of denaturation at 95°C for 30 seconds, hybridization at 59°C for 30 seconds and extension at 72°C for 30 seconds, followed by a final extension at 72°C for 3 minutes. The amplicons were visualized through agarose gel electrophoresis and treated with ExoSAP-IT™ PCR Product Cleanup (Applied Biosystems Thermo Fisher Scientific™) before sequencing.

The sequencing reaction was carried out with the above-described primers and Big Dye Terminator

kit (Applied Biosystems Thermo Fisher Scientific™). Each DNA sequence was determined by capillary electrophoresis with the ABI PRISM® 3500 Genetic Analyzer (Applied Biosystems). The data was analyzed using the software BioEdit Sequence Alignment Editor version 5.0.9, and the sequence of nitrogenous bases of the *BRAF* gene amplicon was identified.

Statistical and diagnostic accuracy analysis

The database was built on the software Microsoft 365 Excel®. For diagnostic accuracy analysis, a 2 x 2 contingency table (true positive, false positive, false negative, and true negative) was generated based on the agreement between the results of diagnostic tests for detecting the BRAF V600E mutation in the ameloblastoma samples using IHC (index test) and Real-Time PCR (reference standard). Sensitivity and specificity measures as well as positive and negative predictive values, with their respective 95% confidence intervals were calculated.

RESULTS

Demographic and clinicopathological characteristics of the sample

The flow diagram of the cases included in the study is shown in Figure 1. Fifty-four FFPE ameloblastoma samples were included. The age at diagnosis varied from 11 to 74 years, with a mean age of 34.48 ± 16.43 years, and the male:female ratio was 1.25:1. Detailed baseline demographic and clinicopathological characteristics of the sample are displayed in Table 1.

Descriptive analysis of the BRAF V600E immunostaining pattern

Positive BRAF V600E immunostaining was identified in 47 out of 54 samples. The immunoexpression pattern was mainly cytoplasmic, distributed throughout both peripheral palisade ameloblast-like and central stellate reticulum-like areas of neoplastic odontogenic epithelium. No stromal immunostaining was observed. Figure 2 illustrates the BRAF V600E immunostaining pattern in different samples.

Background and non-specific staining were observed in wild-type BRAF normal structures adjacent to the tumor site, such as oral mucosa epithelium, salivary gland ducts, and red blood cells, irrespective of tumor mutational status, suggesting the presence of some IHC cross-reactions with tissue immunogens other than specific-BRAF V600E (Figure 3).

Diagnostic accuracy analysis

Although the BRAF V600E immunostaining was observed in 87% of the ameloblastomas, the mutation was detected in only 72.2% of the samples ($n = 39$) by Real-Time PCR (Table 2). Amplification of reference gene detection assay occurred in all samples, confirming the integrity of the *BRAF* gene irrespective of the mutational status.

Sanger Sequencing was carried out in eight cases where there was discordance between the results of Real-Time PCR and IHC. In all instances, the sequencing results were consistent with those obtained from Real-Time PCR. Figure 4 depicts the results of the tests in true positive, true negative, and false positive cases.

The sensitivity and specificity of IHC using specific anti-BRAF V600E antibody (RM8 clone) for the detection of the BRAF V600E mutation in FFPE ameloblastoma samples was 1.00 (95%CI 0.90–1.00) and 0.46 (95%CI 0.21–0.73), respectively, and the diagnostic accuracy was calculated as 85.18%, as shown in Table 2.

DISCUSSION

The recognition of heightened occurrences of the BRAF V600E mutation in ameloblastomas has introduced novel perspectives into the molecular mechanisms that underlie its pathogenesis^{16,17}. Preliminary studies

Table 1. Demographic and clinicopathological characteristics of the sample.

Age at diagnosis	Years	
Age range	11–74	
Mean±SD	34.48±16.43	
	n	%
Sex		
Women	24	44.4
Men	30	55.6
Skin color		
Brown	39	72.2
White	09	16.7
Black	06	11.1
Anatomic location of the tumor		
Mandible	53	98.1
Maxilla	01	1.9
Clinicopathological variant		
Ameloblastoma, conventional	52	96.2
Follicular	20	37.0
Plexiform	16	29.6
Acanthomatous	08	14.8
Basal cell	07	12.9
Desmoplastic	01	1.9
Ameloblastoma, unicystic	01	1.9
Ameloblastoma, adenoid	01	1.9

SD: standard deviation.

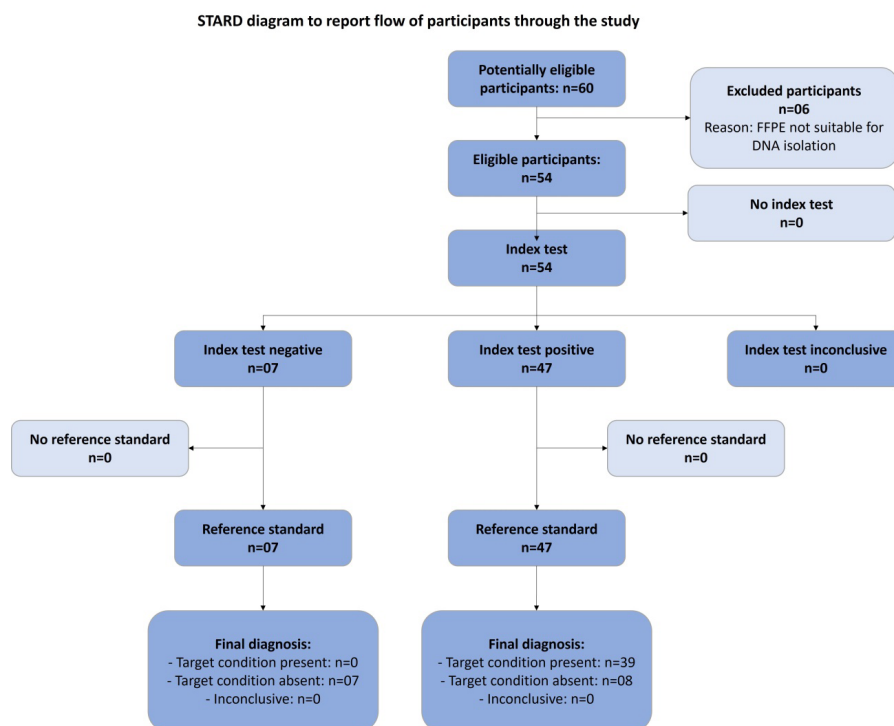


Figure 1. STARD flow diagram of the participants of the study.

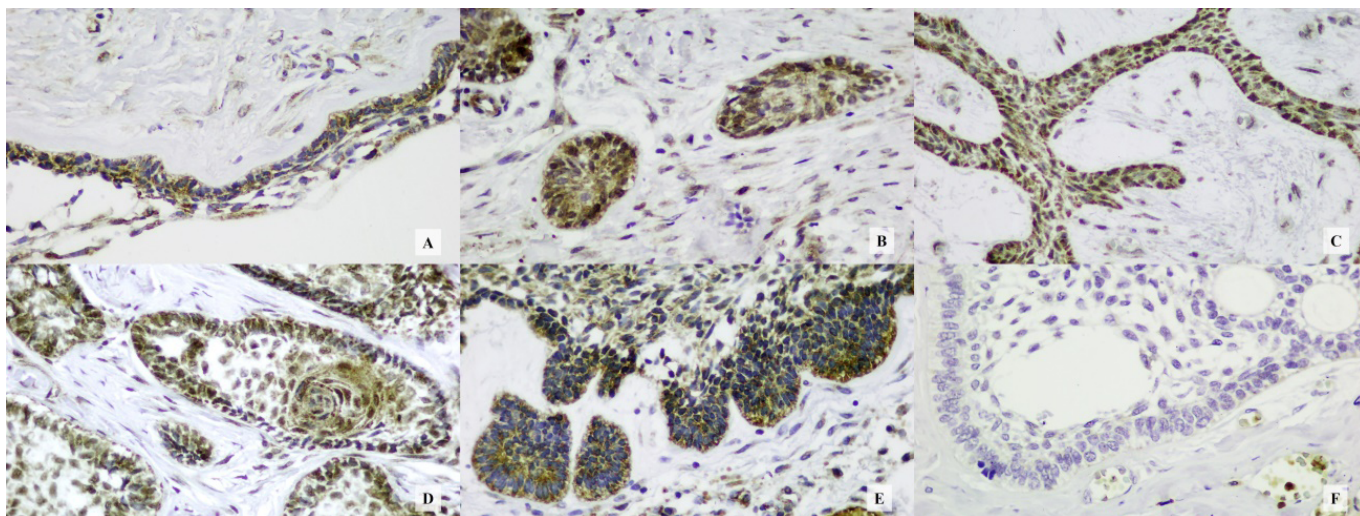


Figure 2. BRAF V600E immunostaining pattern in different ameloblastoma samples. **A**, Positive immunostaining in unicystic ameloblastoma (400x). **B**, Positive immunostaining in follicular conventional ameloblastoma. **C**, Positive immunostaining in plexiform conventional ameloblastoma. **D**, Positive immunostaining in acanthomatous conventional ameloblastoma. **E**, Positive immunostaining in basal cell conventional ameloblastoma. **F**, Negative immunostaining in follicular conventional ameloblastoma.

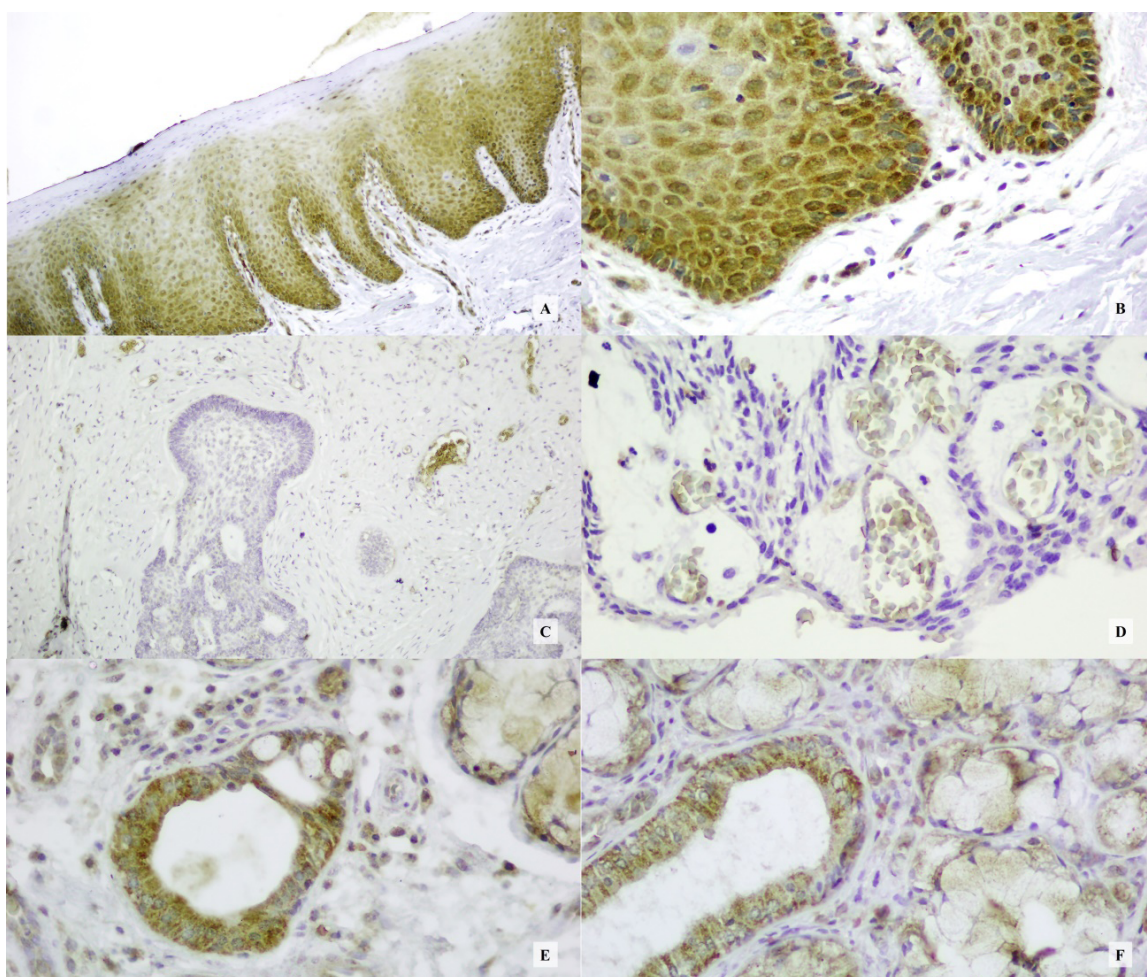


Figure 3. Non-specific immunostaining in wild-type BRAF tissue. **A** (100x) and **B** (400x), Non-specific immunostaining in squamous stratified epithelium of oral mucosa. **C** (100x) and **D** (400x), Non-specific immunostaining in red blood cells. **E** (400x) and **F** (400x), Non-specific immunostaining in salivary gland ducts.

have indicated a favorable response to BRAF-targeted therapies in select cases of ameloblastoma, emphasizing the importance of assessing the BRAF mutational status in these tumors¹⁸⁻²¹. Within this scenario, IHC using mutation-specific antibodies, such as VE1 and RM8 clones, is potentially a less expensive and time-consuming alternative for this purpose in both research and health-care settings⁶. Nevertheless, to the best of the authors' knowledge, this is the first diagnostic accuracy analysis of IHC using anti-BRAF V600E antibody (RM8 clone) in FFPE ameloblastoma samples.

A recent diagnostic accuracy systematic review and meta-analysis conducted by our research team

encompassed five studies demonstrating remarkably high concordance between BRAF V600E-specific IHC using the VE1 clone and molecular reference tests in the detection of the mutation in ameloblastomas⁶. In most of the studies (4/5)^{9-11,13}, both sensitivity and specificity values surpassed 0.90, and no false-positive results were reported for VE1 clone. Contrary to expectations, the present study revealed excellent sensitivity but poor specificity for BRAF V600E-specific IHC using the RM8 clone, with eight false-positive cases.

Likewise, Pereira et al.²² reported four cases of unicystic ameloblastomas and dentigerous cysts with false-positive results using VE1 clone. In these particular

Table 2. Crosstabulation of the index test results by the reference standard results.

		IHC		Accuracy (%)	Sensitivity (95%CI)	Specificity (95%CI)	PPV (95%CI)	NPV (95%CI)
		Positive	Negative					
Molecular tests*	Positive	39	0	85.18	1.00 (0.90–1.00)	0.46 (0.21–0.73)	0.82 (0.75–0.88)	1.00 (0.59–1.00)
	Negative	8	7					

IHC: immunohistochemistry; CI: confidence interval; PPV: positive predictive value; NPV: negative predictive value.

*castPCR™ (all cases) and Sanger Sequencing (discordant cases).

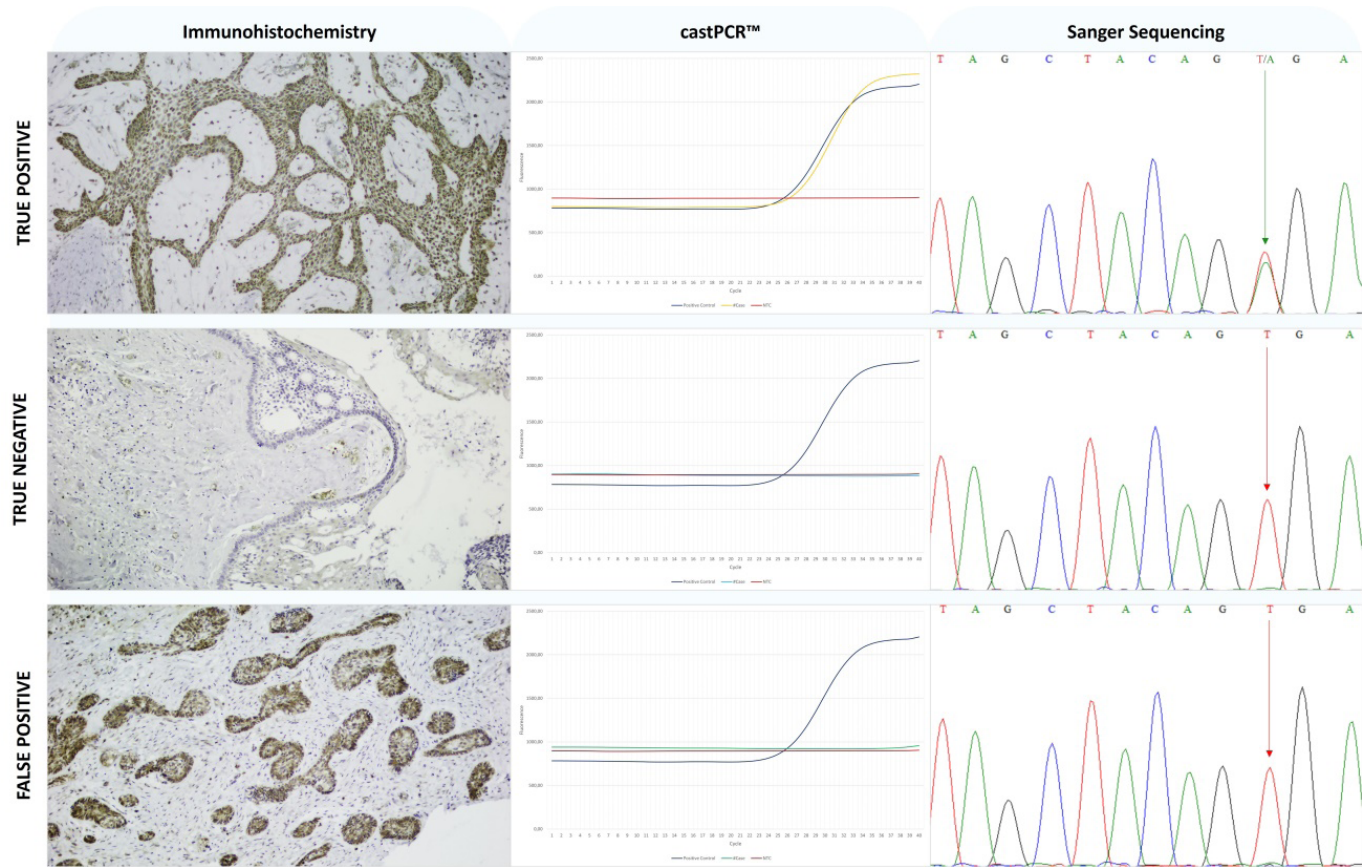


Figure 4. Index test and reference standard results for true-positive, true-negative, and false-positive cases.

cases, the odontogenic epithelium exhibited unequivocal positive immunostaining in confirmed wild-type BRAF samples. However, special attention should be given to cases with divergent IHC and molecular results, as the occurrence of negative cases in real-time PCR may be attributed to the presence of poorly representative tumor samples. This potential limitation underscores the need to consider sample representativeness regarding tumor tissue, which may impact the accuracy of molecular analyses.

Beyond the occurrence of non-specific staining in neoplastic odontogenic epithelium leading to false-positive results, the present study also observed that IHC with RM8 clone produced background and non-specific staining patterns in other wild-type BRAF epithelial and mesenchymal structures, such as oral mucosa epithelium, salivary gland ducts, and red blood cells. While the mechanisms behind these non-specific immunostaining patterns are not completely elucidated, it is plausible that the RM8 clone may exhibit cross-reactivity with other immunogens expressed in these structures. However, the significance of the differences between both VE1 and RM8 monoclonal antibodies in diagnostic accuracy remains to be better explored in future studies.

The most prevalent (> 90%) DNA alteration that results in BRAF V600E mutation is a single nucleotide missense mutation (c.1799T>A) and results in the substitution of valine (V) for glutamic acid (E) in the reading of codon 600. However, other described alteration, c.1799_1800TG>AA, can result in the same amino acid change, consequently producing the same BRAF V600E altered protein²³. Although this protein could be detected by mutation-specific antibodies in IHC, it might be easily missed by the BRAF₄₇₆_mu castPCR™ mutation detection assay. To address this potential issue, the present study employed Sanger Sequencing in all samples with discordant results, hereby providing additional confirmation of the false-positive cases identified by IHC using the RM8 clone.

Even when considering neoplasms other than ameloblastomas, data on the diagnostic accuracy of the RM8 clone remains very limited. In a related study, Yakout et al.²⁴ performed a diagnostic accuracy analysis of IHC using the RM8 clone on a sample of 50 FFPE specimens of melanocytic neoplasms, disclosing suboptimal values for both sensitivity (0.66) and specificity (0.73 to 0.88). This emphasizes the need for further studies involving this monoclonal antibody, as the results can vary substantially across different tumor types, and variations in the index test (IHC technique), such as sample

processing, antigen retrieval, primary antibody dilution, immunostaining system, and positivity threshold may also contribute to different accuracy results^{6,25}.

This diagnostic accuracy study faces some limitations, particularly due to the semi-quantitative nature of the IHC analysis. Determining whether a specific score and cytoplasmic immunostaining pattern indicate positivity requires subjective judgment, and no standardized thresholds for BRAF V600E immunostaining are available in the scientific literature. To minimize the potential bias, we adopted a previously described positivity threshold¹⁵ and two blinded examiners independently assessed the IHC results, with discrepancies resolved through consensus.

CONCLUSION

IHC using BRAF V600E-specific antibody (RM8 clone) showed extremely high sensitivity, but suboptimal (<0.50) specificity when compared with Real-Time PCR in the detection of BRAF V600E mutation in FPPE ameloblastoma samples. Therefore, its feasibility for routine clinical application is still considerably restricted due to the likelihood of false-positive results.

AUTHOR'S CONTRIBUTIONS

AVMB: investigation, methodology, writing – original draft. FACA: formal analysis, funding acquisition. AMIB: data curation. RJGSL: investigation, visualization. AGBN: software, validation. HAMS: conceptualization, resources. MTCM: software. SJF: conceptualization. MVC: funding acquisition, project administration, supervision, writing – review & editing.

CONFLICT OF INTEREST STATEMENT

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Competing interests: The authors have no relevant financial or non-financial interests to disclose.

Ethics approval: This study was approved by the Ethics Committee of the University of Pernambuco (#4.309.512; CAAE: 35920620.7.0000.5192) and all procedures performed in studies involving human

participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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