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Detection of Human Papillomavirus (HPV) and Bovine Leukemia Virus (BLV) in Breast Cancer Patients from Northeastern Brazil

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Abstract: This study evaluated the presence of Human Papillomavirus (HPV) and Bovine Leukemia Virus (BLV) DNA in breast cancer (BC) samples from women in Northeastern Brazil. We analyzed 186 formalin-fixed, paraffin-embedded tissue block samples using PCR and qPCR to detect HPV and BLV DNA. BLV DNA was not detected in any BC patients. However, HPV DNA was found in 46.2% of the BC samples: 44.6% were positive for HPV16, 3.5% for HPV31, and 2% were coinfected with HPV16 and HPV31. Additionally, we found an association between HPV-positive BC and Ki-67 expression (P=0.03, OR=1.85; CI=1.02-3.34), as well as between HPV31-positive BC and mitotic index (P=0.047). Although BLV was not detected in BC patients from Northeastern Brazil, the association between HPV and cell proliferation markers warrants further investigation, as HPV may contribute to the aggressiveness of BC in these patients. 10

Keywords: breast cancer; human papillomavirus (HPV); bovine leukemia virus (BLV); northeast

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1. Introduction

Breast cancer (BC) is a major global health issue. In 2020, the World Health Organization (WHO) reported 2.3 million new cases and 685,000 deaths from BC [1,2]. In Brazil alone, 66,000 women were diagnosed with the disease in the same year [3].

BC is a heterogeneous and multifactorial disease, influenced by both genetic and environmental factors [4–9]. Additionally, studies suggests that infections with Mouse Mammary Tumor Virus (MMTV), Epstein-Barr Virus (EBV), Bovine Leukemia Virus (BLV), and Human Papillomaviruses (HPV) may contribute to its development [6,7,10].

HPV is a well-known cervical cancer agent [11,12]. However, studies have shown the presence of HPV and its putative role in non-cervical cancers, such as head and neck, lung, and breast cancers [5,6,13–19]. Concerning the BC, these studies have shown the presence of HPV DNA 16, 18, 31, 58 and the oncogenes expression of the HPV16 E6, E7 [5,18,20–30]. In addition, the viral load, presence of oncoproteins, and their isoforms were also reported in BC [30–33].

BLV is a retrovirus that infects cattle causing bovine leukosis [34]. BLV is highly prevalent in cattle, and studies suggest that their transmission to humans occurs by ingesting rare meat and unpasteurized milk [34]. In addition, recent studies indicated that BLV could act as a carcinogenic agent in BC since in situ PCR and immunohistochemistry studies detected BLV in BC [34–39].

Despite the existing evidence, the role of HPV and BLV in BC remains controversial, as some studies have failed to detect HPV or BLV DNA in BC samples [35–44]. Given the high prevalence of these viruses in certain regions, such as Northeastern Brazil—where cervical cancer and bovine leukosis are common and where the population consumes rare meat and unpasteurized cow's milk [45–48] it is crucial to conduct further research. Therefore, this study aimed to investigate the presence of HPV and BLV DNA in breast cancer cases from Paraíba State, Northeastern Brazil.

2. Materials and Methods

2.1. Patients

We selected 186 formalin-fixed paraffin-embedded (FFPE) breast cancer (BC) samples from the UNI-LAB Clinical Pathology archives in João Pessoa, Paraíba State, Northeastern Brazil. These samples, collected between 2012 and 2017, included various BC subtypes: 66 (35%) Luminal A, 37 (20%) Luminal B, 31 (17%) HER2-positive, 23 (12%) triple-negative breast cancer (TNBC), 19 (10%) unclassified tumors (NCT), 6 (3%) Super HER2, and 4 (2%) Luminal HER2.

We assessed the presence or absence of HPV DNA in relation to several parameters, including mitotic index, the proliferation marker Ki-67, inflammatory infiltrates, nuclear pleomorphism, tubular differentiation, histological score, vascular invasion, estrogen receptor (ER) status, and progesterone receptor (PR) status. Patient ages ranged from 25 to 91 years. This study was approved by the Ethics and Research Committee under CAAE 4.506.821.

2.2. HPV and BLV Detection

DNA from FFPE BC samples was extracted according to Shi et al., 2002 [49]. The quality of the extracted DNA was verified using polymerase chain reaction (PCR) by amplifying a fragment of the *B*-globin gene with the primers listed in Table 1.

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Table 1. The primers used for conventional PCR and quantitative PCR (qPCR) amplification of HPVand BLV DNA.

	Sequence (5′3′)	Size (bp)
β-globina		
PC04	ACACAACTGTGTTCACTAGC	110 bp
GH20	CAACTTCATCCACGTTCACC	
HPV DNA		
*MY09	CGTCCMARRGGAWACTGATC	450 bp
*MY11	GCMCAGGGWCATAAYAATGG	
HPV DNA		
GP5	TTTGTTACTGTGGTAGATAC	110 bp
GP6	GAAAAATAAACTGTAAATCA	
E6 HPV16	GAGAAACTGCAATGTTTCAGGACC TGTATAGTTGTTTGCAGCTCTGTGC	81 bp
E6 HPV31	CGTTTTCGGTTACAGTTTTACAAGC AGCTGGACTGTCTATGACAT	76 bp
TAX1	CTTCGGGATCCATTACCTG GCTCGAAGGGGGAAAGTGAA	373 bp
TAX2	ATGTCACCATCGATGCCTGG AGCTGGACTGTCTATGACAT	113 bp
GAG	ACCCTACTCCGGCTGACCTA CTTGGACGATGGTGGACCAA	272 bp

* MY09/11 degenerated primers: M = A or C, W = A or T, Y = C or T, and R = A or G.

We used conventional and quantitative real-time PCR (qPCR) to detect BLV and DNA. For HPV detection, we initially employed consensus and degenerate primers MY09/11, followed by nested PCR using oligonucleotides GP05/06 (Table 1) (Table 1) [48,50]. Additionally, we conducted qPCR using the GP05/06 and E6 HPV16-Forward/E6 HPV16-Reverse primers (Table 1) (Table 1) [48]. DNA extracted from CaSki cell lines served a positive control, and all tests were performed in triplicate.

The presence of BLV in BC cases was analyzed using conventional PCR, qPCR, nested PCR, and Auto-Nested qPCR. The primers used targeted the Tax and Gag genes, as de-scribed by Buehring et al. (2014) and Buehring et al. (2019) (Table 1) [51,52]. Proviral DNA extracted from cultured FLK cells containing BLV was used as a positive control.

2.3. Statistical Analysis

We performed chi-square and Fisher's exact tests to compare the clinical aspects of BC patients across the following groups: (i) BC HPV DNA-positive and BC HPV DNA-negative; (ii) BC HPV16 DNA-positive and BC HPV16 DNA-negative; (iii) BC HPV31 DNA-positive and BC HPV31 DNA-negative; (iv) BC BLV DNA-positive and BC BLV DNA-negative. All P-values were two-sided, with a significance level set at 0.05. Data analysis was conducted using SPSS version 26 (SPSS Inc., Illinois, USA).

3. Results

3.1. BLV and HPV DNA Detection in BC

We screened HPV and BLV DNAs in 186 BC cases and did not detect BLV in any of them. On the other hand, we detected HPV DNA in 46.2% (86/186) of the total cases. Among these, we observed 39% (34/86) of HPV DNA in Luminal A, 18% (16/86) in Luminal B, 11% (10/86) in HER-2, 12% (10/86) in TNBC, 13% (11/86) in NCT, 2% (2/86), in Super HER2 and 2% (2/86) in

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Luminal HER2. Although we detected HPV DNA in all molecular types of BC, no significant differences were observed between BC HPV DNA-positive cases and molecular types (Table 2). In addition, we did not find significant differences between BC HPV DNA-positive and mitotic index, inflammatory infiltrate nuclear pleomorphism, tubular differentiation, histological score, vascular invasion, ER, and PR (Table 2). However, BC HPV DNA-positive cases are associated with the Ki-67 expression (Table 2). Finally, we did not observe statistical differences between Luminal vs. Non-Luminal (p= 0.338) and 86 Luminal A vs. Luminal B (p= 0.281) in BC HPV DNA-positive cases.

Table 2. Clinical and histological characteristics of patients according to HPV status in the BC.

		BC-HPV positive N°(%)	BC-HPV negative N°(%)	*p*-value	OR (IC)
Age	≤ 25	1 (14.28%)	6 (85.71%)	0.08	
	26 – 40	27 (53%)	24 (47%)		
	41 – 60	46 (50%)	46 (50%)		
	> 60	12 (33.33%)	24 (66.67%)		
Tumor localization	Right	41 (44.57%)	51 (55.43%)	.817	
	Left	45 (48.39%)	48 (51.61%)		
	Both	0 (0%)	1 (100%)		
Histopathological types	TNE/SOE	71(47.65%)	78 (52.35%)	.450	
	CDI	6 (42.86%)	8 (57.14%)		
	CDIISBR	2 (100%)	0 (0%)		
	CDINV	6 (35.2%)	11 (64.7%)		
	CDL	0 (0%)	1 (100%)		
	CINADER	0 (0%)	1 (100%)		
	CILOB	1 (100%)	0 (0%)		
Molecular types	Luminal A	34 (51.52%)	32 (48.48%)	.287	
	Luminal B	16 (43.24%)	21 (56.76%)		
	HER2-Positive	10 (32.26%)	21 (67.74%)		
	TNBC	10 (43.48%)	13 (56.52%)		
	NCT	11 (68.75%)	5 (31.25%)		
	Super HER2	2 (33.33%)	4 (66.67%)		
	Luminal HER2	2 (33.33%)	4 (66.67%)		
RE expression	Yes	69 (49.2%)	71 (50.7%)	.174	
	No	17 (37%)	29 (63%)		
PR expression	Yes	28 (40%)	42 (60%)	.225	
	No	58 (50%)	58 (50%)		
Ki-67 expression	Ki-67<30	73(50.3%)	72 (49.7%)	.035	Ki-67>30 1,852 (1,025-3,34)
	Ki-67>30	13 (6.09%)	28 (15%)		
Mitotic index score	ND	2 (66.67%)	1 (33.33%)	.467	
	I	27 (48.21%)	29 (51.9%)		
	II	19 (65.52%)	10 (34.48%)		
	III	9 (50%)	9 (50%)		
Inflammatory index	IIEA	10 (71.43%)	4 (28.57%)	.842	
	IILPE	0 (0%)	1 (100%)		
	IINPM	1 (50%)	1 (50%)		
	INPA	12 (48%)	13 (52%)		
	E	8 (61.54%)	5 (38.46%)		
	M	0 (0%)	1 (100%)		
	I	1 (50%)	1 (50%)		
	A	11 (61.11%)	7 (38.89%)		
	L	2 (50%)	2 (50%)		
	IITD	1 (50%)	1 (50%)		
	ILETM	1 (100%)	0 (0%)		

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	INPP	10(43.48%)	13 (56.52%)	
Nuclear Pleomorphism score	ND	2 (66.67%)	1 (33.33%)	.639
	I	0 (0%)	1 (100%)	
	II	16 (48.48%)	17 (51.51%)	
	III	39 (56.52%)	30 (43.48%)	
Tubular differentiation score	ND	1 (100%)	0 (0%)	.252
	I	1 (20%)	4 (80%)	
	II	13(48.15%)	14 (51.85%)	
	III	42 (39.62%)	31 (60.38%)	
Histological score	ND	5 (62.5%)	3 (37.5%)	.646
	I	4 (36.36%)	7 (63.63%)	
	II	29 (54.72%)	24 (45.28%)	
	III	19 (57.58%)	14 (42.42%)	
Vascular invasion	ND	8 (47.06%)	9 (52.94%)	.796
	Present	14 (51.85%)	13 (48.15%)	
	Absent	35 (56.45%)	27 (43.55%)	

Data are presented as the number of patients and percentage. p-values were calculated by chi-square test or Fisher's exact test. OR = Odds Ratio. *p<0.05-Statistical significantly. Inflammatory infiltrate IIEA: Absent tumor stromal inflammatory infiltrate; IILP E: Peritumoral lymphoplasmacytic inflammatory infiltrate scarce; INPM: Moderate peritoneal peritumoral neoplastic infiltrate; INPA: Absent peritoneal inflammation; E: Scarce; M: Moderate; I: Intense; A: Absent; L: Lightweight; IITD: Discrete tumor inflammatory infiltrate; ILETM: Lymphocytic infiltrates in the minimal tumor stroma; INPP: Present perineural inflammation. Mitotic index: I: measures the speed with which tumor cells expand and segment; II and III are related to the incidence of axillary metastasis. Degree of nuclear polymorphism; I: Small, regular, and uniform tumors; II: points with a moderate increase in size and variability; III: Significant variation; Degree of tubular differentiation: I: Most of the tumor (>75%); II: Moderate part of the tumor (10-75%); III: Little or nopart of the tumor (<10%); Histological grade: I: cells similar to normal and slow growing; II: cells that look less like normal and have moderate growth; cells distinct from normal and fast-growing cells; Vascular invasion: Present: there is evidence of invasion of the vascular or lymphatic system and Absent: There was no evidence of lymphatic invasion.

3.2. HPV16 and 31 DNA Detection in BC

The most frequent HPV type in the BC cases was HPV16 (44.6%, 83/186). It was also the most common type in Luminal A, Luminal B, HER2, TNBC, and Super HER2. However, despite its high prevalence, we did not observe significant differences between BC HPV16 DNA-positive cases and molecular types, mitotic index, inflammatory infiltrate, nuclear pleomorphism, tubular differentiation, histological grade, vascular invasion, ER, PR, and Ki-67 (Table 1 of the supplementary data). Furthermore, we did not find significant differences between Luminal vs. Non-Luminal groups and BC HPV16 DNA-positive cases (p=0.636).

We found 3.8% (7/186) of HPV31 in all BC patients, specifically in Luminal A and TNBC cases. When comparing Luminal vs. Non-Luminal types, no significant differences were observed with BC HPV31 DNA-positive cases (p=0.269). Additionally, there were no significant differences between BC HPV31 DNA-positive cases and molecular types, inflammatory infiltrate, nuclear pleomorphism, tubular differentiation, histological grade, vascular invasion, ER, PR, and Ki-67 expression (Table 2 of the supplementary data). However, a significant association was found between the mitotic index and BC HPV31 DNA-positive cases (p=0.047). Coinfections with HPV16 and HPV31 genotypes were observed in 2% (4/186) of the total BC samples.

4. Discussion

This study investigated the presence of BLV, HPV16, HPV31, and their coinfections in BC samples from women in Northeast Brazil. We did not detect BLV in any BC samples. However, HPV DNA was found in 46% of the samples, with HPV16 present in 44% of the tumors. Despite

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the high prevalence of HPV, there was no association between HPV, HPV16, or HPV31 DNA and molecular types, inflammatory infiltrate, nuclear pleomorphism, tubular differentiation, histological grade, vascular invasion, ER, or PR. We did observe an association between HPV and cell proliferation markers.

Diverse studies have demonstrated the presence of HPV DNA in BC, showing detection in both fresh and FFPE tissues using various methodologies such as IHC, conventional PCR, qPCR, and in situ PCR. [18,20–24,27–30,40,53–58]. In addition, these studies detected HPV DNA using different methodologies, such as IHC, conventional PCR, qPCR, and in situ PCR [18,20–24,27–30,40,52–57]. These studies reported HPV DNA prevalence in BC ranging from 0% to 71%, influenced by detection methods and sample processing techniques [18,20–24,27–30,40,52–57]. The methodology used to detect HPV DNA and the sample processing with fixation may reflect the prevalence variation in BC. Our study found a prevalence of HPV DNA in 46% of BC patients from Northeastern Brazil, where cervical cancer is a major public health concern [45–48,59,60]. Among these samples, 44% were infected by HPV16 and 3.8% by HPV31. Coinfection with HPV16 and 31 was found in 1.6% of total samples. Some studies found similar results in a different population, where HPV16 is most prevalent and HPV31 is less common [23,29,60]. HPV 16 and HPV 31 are the most frequent genotypes found in cervical cancer in women from Northeastern Brazil. However, this coinfection was less frequent in BC patients. We used conventional PCR and qPCR to detect HPV DNA in BC samples. Although PCR and qPCR did no to detect multiple HPV types simultaneously, this methodology suits HPV DNA detection.

Among BC patients, HPV DNA was found most frequently in Luminal patients, which contrasts with other studies showing a higher frequency in TNBC and HER-2 patients [24,61,62]. Although HPV DNA was most prevalent in Luminal patients, specifically Luminal A, these frequencies were not statistically significant. Similarly, HPV DNA was most frequent in PR and ER expression, but these differences were not statistically significant. However, we found a significant association between Ki-67 expression and HPV DNA in BC, as well as between the mitotic index and BC HPV31 positivity. Studies by de Carolis et al. (2019) also showed a similar association between BC HPV DNA-positive status and Ki-67 in Luminal B patients [24]. Both the Ki-67 marker and the mitotic index measure cell proliferation, suggesting that BC HPV DNA-positive cases may be more aggressive than BC HPV DNA-negative cases. Future studies with larger case-control samples are needed to clarify the role of cell proliferation markers in BC patients infected by HPV.

This study did not detect BLV in BC patients from Northeastern Brazil. Previous investigations using various methodologies, including in situ PCR, qPCR, and sequencing to amplify LTR, gag, pol, env, or tax genes, also observed the absence of BLV in BC [62,63]. However, some studies have reported the presence of BLV in BC patients from different regions worldwide, including Brazil [51,52,64–69]. The discrepancy between our findings and those of other studies may be attributed to different environmental factors and methodologies used to detect BLV. Environmental factors such as lower meat consumption and coinfections with different viruses could explain these differences. In this study, we used conventional PCR, qPCR, Nested PCR, and AutoNested PCR to amplify gag and tax gene fragments. Interestingly, we found a high prevalence of HPV and an absence of BLV, whereas a study by Baltzell et al. (2017) found BLV but no coinfection with HPV [65]. Future studies should aim to clarify the relationship between BC and coinfections with common local viral agents, such as HPV and BLV.

5. Conclusions

This study identified HPV16 and HPV31, but not BLV, in BC patients. Additionally, we found a significant association between BC HPV-positive cases and cell proliferation markers. Future research should aim to elucidate the relationship between cell proliferation markers and BC HPV DNA-positive cases in the context of carcinogenesis.

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Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Table S1: Clinical and histological characteristics of patients according to HPV16 status in the BC. Table S2: Clinical and histological characteristics of patients according to HPV31 status in the BC.

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