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Comparative study of five commercial probes for the detection of Epstein-Barr virus (EBV) by *in situ* hybridization in cases of nodular sclerosis Hodgki's lymphoma

Estudo comparativo de cinco sondas comerciais para detecção do vírus Epstein-Barr (EBV) por hibridização in situ *em casos de linfoma de Hodgkin esclerose nodular*

Suely Nonogaki; Neuza K. Shirata; Lidia M. Kimura; Juliana M. Guerra; Raphael S. S. Medeiros; Roberto Antonio P. Paes; Cristina T. Kanamura; Camila C. Oliveira; Yara de Menezes

Instituto Adolfo Lutz, São Paulo, Brazil.

ABSTRACT

Introduction: Epstein-Barr virus (EBV) may serve as a target in therapeutic treatments, thus reliable diagnostic results are necessary. **Objective:** The aim of this study was to evaluate the accuracy of EBV detection by *in situ* hybridization (ISH) using five commercial probes in formalin-fixed and paraffin-embedded samples of nodular sclerosis Hodgkin's lymphoma (HL), and to compare the results with immunohistochemistry (IHC) and polymerase chain reaction (PCR). **Material and method:** Thirty samples were selected, 28 were lymph nodes, one bone marrow and one mediastinum. The following parameters were analyzed: signal intensity; proportionality of positive cells; quality of the reaction according to comfort for evaluation, sign quality and homogeneity of labeled cells; background reaction; morphology; presence of artifacts; and positivity in other non-neoplastic cells. All samples were analyzed for EBV detection using the five probes, IHC for latent membrane protein type 1 (LMP1) and PCR for Epstein Barr virus nuclear antigen 1 (EBNA1). Statistical analyses were performed with the R1 software; Fleiss' test and Cohen Kappa index of 5% were considered significant. **Results:** The detection by IHC-LMP1 was 26.7% (8/30) and 66.7% (20/30) by PCR-EBNA1. All probes detected EBV. Positivity was observed in 42/90 (46.7%), 38/90 (42.2%), 45/90 (50%), 27/90 (30%) and 61/90 (67.8%) for probes A, B, C, D and E, respectively. **Discussion:** All five probes demonstrated positivity. **Conclusion:** Probe E showed better rate (67.8%), sensitivity, specificity and accuracy (100%), a very good correlation among the different observers and with PCR, besides great cost-benefits relation.

Key words: Epstein-Barr virus infections; *in situ* hybridization; Hodgkin disease.

INTRODUCTION

Epstein-Barr virus (EBV) belongs to the human herpes virus family broadly spread to worldwide. Epidemiological studies have estimated that 90%-95% of the human adult population are infected with EBV[1-6] and has been transmitted intermittently through saliva. In most individuals, this virus promotes an asymptomatic to subclinical infection[3, 6] and persists latently in the host. This agent was the first tumor viruses discovered in humans and is associated with various types of neoplasms such as undifferentiated nasopharyngeal carcinoma, Burkitt lymphoma, Hodgkin lymphoma (HL), nasal lymphoma of natural killer and T cells (NK/T) and gastric carcinoma[3, 5, 7, 8].

EBV is a linear, double-stranded deoxyribonucleic acid (DNA) virus with 172,000 base pairs, which can remain latent in the lymphoid cells in its episomal form[3, 5]. These cells express genes, among others, a small non-polyadenylated ribonucleic acid (RNA) chain that does not translate a protein, consisting of two fragments known as EBER1 (166 nucleotides) and EBER2 (172 nucleotides). The expression of both EBER [Epstein-Barr Encoded

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(Early) RNAs] is nuclear, which may suggest their involvement in replication, transcription or RNA processing in cells transformed by EBV. Despite the EBER function has not being completely clarified, some authors suggest that EBER molecules promote the survival of both the host cell and the viral growth[9] and may play a role in the oncogenesis ability[2].

HL is a singular neoplasm, histologically characterized by the presence of malignant cells called Reed-Sternberg (R-S) cells. Currently, HL are classified as: nodular lymphocytic predominance and classic lymphoma, the latter is subdivided into nodular sclerosis (HLNS), rich in lymphocytes, mixed cellularity and lymphocyte depletion.

HLNS, initially described by Lukes, Butler and Hicks in 1964, is characterized by nodular pattern separate by bundles of collagen and lacunar cells[10]. This is the most common subtype of HL and has distinct epidemiological characteristics, it is more frequent among young female adults, residents of economically developed regions and less frequently associated with EBV[11].

Some of the methods that can detect EBV in formalin-fixed and paraffin-embedded samples, are immunohistochemistry (IHC) for latent membrane protein type 1 (LMP1), polymerase chain reaction (PCR) and *in situ* hybridization (ISH). LMP1 is a transmembrane protein with 63-kDa phosphoprotein encoded by BNLF1 gene and is found in several EBV-associated malignancies[3]. Comparative studies showed that the PCR is the most sensitive method, followed by ISH and IHC, however, the PCR can not indicate the location of cells infected by the virus[1, 12, 13]. In addition, Gulley and Tang[8], point to EBER-ISH transcripts as the gold standard for identifying EBV latent infection.

The aim of this study was to evaluate the accuracy of EBV detection by ISH, using five different commercially available probes, in São Paulo, Brazil, in cases of HLNS, and compare it to the results of IHC and PCR.

MATERIAL AND METHOD

Material

Thirty consecutive samples of nodular-sclerosis Hodgkin lymphoma's subtype were selected among those received by the Pathology Center of Adolfo Lutz Institute between 2009 and 2010, based on immunohistochemistry analysis results reports. Twenty eight samples were from lymph nodes, one bone marrow and one mediastinum. All samples were reviewed according to the World Health Organization (WHO)[14] criteria, in consensus, by three pathologists (RAPP, RSSM, YM). All samples were analyzed for the detection of EBV-EBER using the five probes and IHC-LMP1 and PCR-EBNA1.

The project was approved by the Institutional Review Board of the Adolfo Lutz Institute (CTC-IAL) under nº 95629/2011 and Ethics Committee (CEP-IAL) under nº 027/2011.

Immunohistochemistry

The IHC reactions were carried out at the Immunohistochemistry Laboratory-Pathology Center at the Adolfo Lutz Institute, as a routine diagnostic procedure. Briefly, after removing paraffin residues, the tissue sections (3 µm) fixed on silanized glass slides were submitted to antigen retrieval procedure, performed in a stainless steel pressure cooker (Evinox 2005, Cruzinox Ind. Metalurgica, Carregosa, Portugal) using 10 mM citric acid solution/pH 6.0 (Merck, 244, Darmstadt, Germany) for three minutes under pressure[15]. Endogenous peroxidase activity was blocked by incubating with 6% aqueous hydrogen peroxide solution (Quimesp, P011.09.201, Guarulhos, Brazil) for 20 minutes. The primary antibody used in this study was a mouse EBV LMP monoclonal antibody, clones CS1-4 cocktail (Dako, M0897, Glostrup, Denmark) diluted 1:400 in phosphate buffer saline (PBS) with 1% bovine serum albumin (BSA) Cohn fraction V (Inlab, 1870, São Paulo, Brazil), in overnight incubation at 4ºC in humidified chamber. The antigen-antibody link was amplified by a third generation polymer tagged with anti-mouse and anti-rabbit immunoglobulins and horseradish peroxidase (HRP) (Novolink Polymer Detection System, Leica Biosystems, Newcastle Upon Tyne, UK)[16] and the color developed with diaminobenzidine (DAB) chromogenic substrate (Sigma, D5637, St. Louis, MO, USA).

Positive cells were observed in golden brown color. The negative control was performed by omitting the primary antibody.

In situ hybridization

For ISH reactions, five new histological sections (5 µm) were obtained, on a rotary microtome (Leica RM2245, Leica Mycrosystems, Nusslock, Germany) under aseptic conditions to avoid cross-contamination; from 30 paraffin samples (150 slides) and applied to silanized slides. In addition to the 30 samples, others confirmed by IHC to cytomegalovirus (CMV), herpes simplex (HSV) and human herpes virus 8 (HHV-8) were included to assess the specificity of the probes.

Positive controls were included in all runs. The negative control was carried out by RNA negative probe. Samples followed individualized protocols for ISH probes, according to the package

inserts, but equaling the volume of the probes. **Table 1** displays the protocols by probe. All ISH procedures were performed by one person (SN).

Probe A

Histosonda EBER (Cenbimo, CEM-0001, Lugo, Spain). It is a single-stranded DNA with 526 nucleotides complementary to EBER that detects the gene in its entirety, including EBER1 and 2. Since it has a greater length, the annealing forms only to its target sequence. Its technology is primarily based on incubation at 62ºC for a short time (1 hour), which allows only the chain with complete homology to perform the annealing, and which would not occur with oligonucleotides at that temperature. The wash buffer used was PBS and formamide is not required. All products, except the proteinase K, can be stored at room temperature. The detection system is not supplied. The one-step polymer SuperPicture third-generation Poly-HRP Conjugate (Invitrogen, 87-8963, Camarillo, CA, USA) was incubated for 30 min at 37ºC and developed with DAB Chromogen and Substrate Buffer (Novocastra, RE7162 and RE71630, Leica Biosystems, Newcastle Upon Tyne, United Kingdom) diluted 1:20 and incubated for 10 min at room temperature. Positive samples showed golden brown nuclear staining.

Probe B

EBV (EBER) peptide nucleic acid (PNA) probe/fluorescein (DakoCytomation, Y5200, Glostrup, Denmark) and PNA ISH detection kit (DakoCytomation, K5201). It is a mixture of four fluorescein-labeled PNA probes complementary to part of two nuclear EBER RNAs. The detection kit contains proteinase K, positive (fluorescein-conjugated PNA probe directed against glyceraldehyde 3-phosphate dehydrogenase in hybridization solution) and negative (fluorescein-conjugated random PNA probes in hybridization solution) controls probes, wash solution, alkaline phosphatase-conjugated rabbit fraction F (ab') anti fluorescein isothiocyanate (FITC), chromogenic substrate combined with inhibitor of endogenous alkaline phosphatase (BCIP/NBT/Levamisole) and Tris buffer. The hybridization was performed at 55ºC for 1 hour and 30 min. Positive samples showed purple nuclear staining.

Probe C

Fluorescein-conjugated EBV Probe (Novocastra NCL-EBV, Leica Biosystems) and Novocastra Universal ISH Detection Kit. It is a fluorescein-labeled oligonucleotide cocktail for detection of mRNA sequence, showing latently infected cells, hybridizing

TABLE 1 – ISH – protocols by probes

	Probe A	Probe B	Probe C	Probe D	Probe E
Prehybridization	Denaturation at 62ºC for 10 min Deparaffinization in xylene Decreasing concentrations of ethanol Wash. Deionized water Endogenous peroxidase block. Methanol + 6% H_2O_2 v/v. 5 min Nonspecific DNA inhibition. 30 s Deproteinisation. Proteinase K. 5 min. Room temperature Wash. Deionized water Wash. PBS	Deparaffinization in xylene Decreasing concentrations of ethanol Wash. Deionized water Endogenous peroxidase block. 6% H_2O_2 5 min Deproteinisation. Proteinase K. 10 min. Room temperature Wash. Deionized water Ethanol 95% – Air dry	Deparaffinization in xylene Decreasing concentrations of ethanol Wash. Deionized water Endogenous peroxidase block. 6% H_2O_2 5 min Deproteinisation. Proteinase K. 10 min. Room temperature Wash. Deionized water Ethanol 95% and 100% – Air dry	Deparaffinization in xylene Decreasing concentrations of ethanol Wash. Deionized water Endogenous peroxidase block. 6% H_2O_2 5 min Wash. Deionized water Ethanol 95% and 100% – Air dry Desproteinisation. Preheated pepsin. 37ºC for 5 min Wash. Deionized water Ethanol 95% and 100% – Air dry Denaturation. 60ºC for 5 min	Denaturation at 70ºC for 10 min Deparafinization in xylene Decreasing concentrations of ethanol Wash. Deionized water Endogenous peroxidase block. Methanol + 6% H_2O_2 v/v. 5 min Wash. Deionized water Deproteinisation. Pepsin. 3 min for 37ºC Wash. Deionized water Heat Pretreatment Solution EDTA at 95ºC for 15 min Rinse. Deionized water
In situ hybridization	Vortex the vial for 30 s Probe – Single-stranded DNA with 526 nucleotides complementary to EBER (mRNA) labeled with digoxigenin. Hybridization at 62ºC for 1 h.	Vortex the vial for 30 s Probe – PNA fluorescein labeled. Mix of 4 PNA probes complementary to part of both nuclear EBER RNA sequence. Hybridization at 55ºC for 1h30min.	Vortex the vial for 30 s Probe – Mix of oligonucleotides labeled with fluorescein complementary to nuclear mRNA sequence. Hybridization at 37ºC. Overnight.	Vortex the vial for 30 s Probe – Mix of 5 oligonucleotides complementary to the EBER, labeled with digoxigenin. Hybridization at 37ºC. Overnight.	Vortex the vial for 30 s Probe – Mix of 5 oligonucleotides complementary to the EBER, labeled with digoxigenin. Denaturation at 75ºC for 5 min. Hybridization at 55ºC for 1 h.
Posthybridization & detection	Rinse. PBS Mouse conjugated antidigoxigenin. 37ºC for 30min Rinse. PBS Third-generation polimer conjugated to HRP. 37ºC for 30 min Rinse. PBS Substrate solution. DAB. 10 min at room temperature Wash. Deionized water Hematoxylin. 10 s Wash. Deionized water Differentiation. 3 s Wash. Deionized water Ascending concentrations of ethanol Xylene Entellan Neu	Preheated wash solution. 55ºC for 25 min Rinse. TBS Rabbit anti-FITC labeled with alkaline phosphatase. 37ºC for 30 min Rinse. TBS Rinse. Deionized water Substrate solution. NBT/BCIP. 37ºC for 1 h Rinse. Deionized water Hematoxylin. 10 s Wash. Deionized water Differentiation. 3 s Wash. Deionized water Aquatex	Rinse. TBS Wash solution I = TBS + 0.1% Triton X-100 Blocking solution = solution I + 3% BSA + 20% Normal rabbit serum. 10 min. Room temperature Discard the blocking solution Rabbit conjugated F (ab') anti-FITC labered with alkaline phosphatase diluted 1:50 in solution I + 3% BSA. 37ºC for 30 min Rinse. TBS Rinse. Alkaline phosphate buffer pH 9 Substrate diluted 1:50 + 1 µl levamisole for each 1 ml. 37ºC for 1 h Wash. Deionized water Hematoxylin. 10 s Wash. Deionized water Differentiation. 3 s Wash. Deionized water Aquatex	Wash. TBS Anti-digoxigenin labeled with alkaline phosphatase. 37ºC for 30 min Rinse. TBS Rinse. Deionized water Substrate solution. NBT/BCIP. 37ºC for 1 h Wash. Deionized water Nuclear fast red. Room temperature for 5 min Wash. Deionized water Aquatex	Rinse. TBS Rinse. TBS. 55ºC for 5 min Rinse. TBS. Room temperature for 5 min Mouse conjugated anti-digoxigenin. 37ºC for 30 min Rinse. TBS Polimer conjugated to HRP. 37ºC for 30 min Rinse. TBS Substrate solution. DAB. Room temperature for 10 min Wash. Deionized water Hematoxylin. 10 s Wash. Deionized water Differentiation. 3 s Wash. Deionized water Ascending concentrations of ethanol Xylene Entellan Neu

ISH: in situ *hybridization; v/v: volume for volume; EDTA: ethylenediamine tetraacetic acid; DNA: deoxyribonucleic acid; EBER: Epstein-Barr encoded; mRNA: messenger ribonucleic acid; PNA: peptide nucleic acid; PBS: phosphate buffered saline; TBS: tris-buffered saline buffer; FITC: fluorescein isothiocyanate; HRP: horseradish peroxidase; BSA: bovine serum albumin; NBT: nitro blue tetrazolium chloride; BCIP: 5-Bromo-4-chloro-3-indolyl-phosphate; DAB: 3,3'diaminobenzidine.*

to the EBER concentrated in their nuclei. The detection kit contains rabbit fraction F (ab') anti FITC conjugated to alkaline phosphatase, enzyme substrate and the alkaline phosphatase inhibitor. The hybridization occurs at 37ºC. Positive samples showed purple nuclear staining.

Probe D

EBV small RNA's probe (PROBE XXXEBER) (PanPath, A500P.9900) and DIG-AP Rembrandt Universal RISH & Detection Kit (PanPath A000K.9905, Amsterdam, The Netherlands). It is a mix of 5 oligonucleotides complementary to the region encoding the EBER, labeled with digoxigenin. The kit for detection contains the enzyme to proteolytic treatment, anti-digoxigenin antibody labeled to alkaline phosphatase, positive and negative controls probes, the chromogenic substrate and the wash solution. The recommended hybridization temperature is 37ºC overnight incubation. The counterstaining is performed with Nuclear Fast Red. Positive samples showed purple nuclear staining.

Probe E

ZytoFast EBV Probe (ZytoVision T-1114-400) and ZytoFast Plus CISH Implementation HRP-DAB kit (ZytoVision T-1063, ZytoVision GmbH, Bremerhaven, Germany). It is a digoxigenin-labeled oligonucleotide, which links to an anti-digoxigenin antibody and subsequently to a polymer conjugated to an enzyme and developed with DAB. The detection kit contains positive [consists of poly-dT oligonucleotides targeting the poly (A) tails of mRNAs] and negative [consists of a set of random oligonucleotides sequence with guanine-cytosine (GC) contents of 40%-70% without known consensus to any naturally occurring sequences] controls probes for DNA and RNA, reagents for pretreatment, wash buffer, anti digoxigenin antibody, polymer conjugated to peroxidase, chromogen, chromogenic substrate and embedding agent. The recommended denaturing temperature is 75ºC and hybridization at 55ºC. The incubation with antibody and polymer were carried out at 37ºC. Positive samples showed golden brown nuclear staining.

Polymerase chain reaction

Isolation of the genomic DNA aseptically obtained from 20 µm of paraffin tissue blocks was confirmed using a Maxwell 16 AS2000 instrument and the Maxwell 16 FFPE Plus LEV DNA Purification Kit (Promega Corporation, Madison, WI, USA). The purity of the isolates and DNA concentration was measured using QuantiFluor dsDNA System and Quantus Fluorometer (Promega Corporation, Madison, WI, USA).

EBV genotyping was performed by nested-PCR and the DNA presence was verified by amplification of the β-globin gene. The first PCR reaction amplified 269-base pairs (pb) of common region of Epstein Barr virus nuclear antigen 1 (EBNA1) with the primers EBNA-1F[7] (from 5' to 3'): gTC ATC ATC ATC Cgg gTC TC and EBNA-1R : TTC ggg TTg gAA CCT CCT Tg and a simultaneous 110-bp segment of β-globin gene with the following primers[17] (from 5' to 3'): PCO3: ACA CAA CTg TgT TCA CTA gC; PCO4: CAA CTT CAT CCA CgT TCA CC. PCR was performed in a final volume reaction of 50 µl containing 300 ng of DNA and FastStart PCR Master (Roche Molecular System, Inc., Brachburg, NJ, USA).

After the first amplification, 5 µl was transferred from the first to the second run mixture with inner primers EBNAI-1F (5' CCT CCA ggT AgA Agg CCA 3') and EBNAI-1R (5' ACC ACg ATg CTT TCC AAC C 3'), which amplified 216-bp fragment designed with primer 3 (http://bioinfo.ut.ee/primers3-0.4.0/). The following thermal cycle conditions were used for both runs: 94ºC for 5 min, 40 cycles of 94ºC for 45 s, 55ºC for 45 s, 72ºC for 45 s, followed by 72ºC for 5 min in Eppendorf Mastercycler pro PCR thermal cycler (Eppendorf, Hamburg, Germany).

Blood sample from an EBV carrier and sterile distilled water were used as positive and negative controls, respectively. PCR products were visualized with ultraviolet light as a single band by staining with DNA Blue Green loading dye 1X (LGC Biotecnologia, São Paulo, Brazil) after 2% agarose gel electrophoresis.

Data analysis

Three pathologists, two hematopathologists and one general pathologist, as a blinded study, evaluated the slides labeled with the five probes.

Each pathologist examined the following parameters: the signal strength (intensity); proportionality of labeled cells; reaction quality in positive samples such as comfort for evaluation, signal quality and homogeneity of labeled cells; background reaction; morphology; positivity in other non-neoplastic cells and the presence of artifacts.

The signal strength was classified as 0 = none, 1 = weak, 2 = intermediate and 3 = strong. The proportion of positive cells was classified as 0 = none; 1 for < 1/100; 2 = 1/100 to 1/10; 3 = 1/10 to 1/3; 4 = 1/3 to 2/3; and 5 for > 2/3. The Allred Score[18] was determined by the sum of the two ratings, resulting in a score that ranged from 0, 2-8. Zero was negative and 2 or more was considered positive.

The reaction quality included the sum of the comfort for evaluation, the signal quality and homogeneity of labeled cells,

and were classified by pathologists into 1 = bad and 2 = good, resulting in a score of 3-6.

The morphology was classified as 0 = unanalysable, 1 = no cell recognition, 2 = difficulty in cell recognition, and 3 = good cell recognition.

The background reaction, the presence of artifacts and the positivity in non-neoplastic cells other than Reed-Sternberg were classified as 0 and 1 when absent and present, respectively.

Statistical analysis

The absolute frequencies of the classes were determined for each parameter. The agreement was classified according to Fleiss' Kappa. It was considered positive for ISH reaction only when there was a consensus between at least two observers.

The performance and ease of execution, the run time and the cost of reaction were also evaluated. Kappa test was used to compare the frequencies obtained by the ISH, IHC and PCR, and the calculations for specificity, positive and negative predictive value, false positive rate, and accuracy were also performed.

Analyses were performed in the R1 software, Minitab (Minitab Inc, PA, USA), and in this study the significance level of 5% was considered.

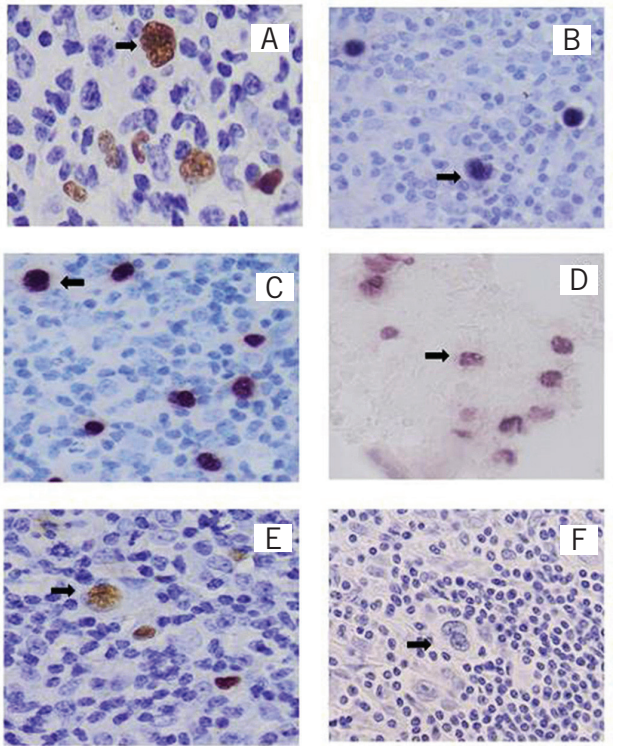

FIGURE – *ISH*
Sample number 30 (lymph node) labeled by the five probes A-E. A-E) positive cells are labeled in brown or purple (bold arrows); F) RNA negative probe. Original magnification 400× from A to F.

ISH: in situ *hybridization; RNA: ribonucleic acid.*

RESULTS

Among the 30 samples, 18 were from males and 12 from females. The average age was 29.4 [standard deviation (SD) = 14.9] years old. Eight (26.7%) samples were from patients under 18 years old and 22 (73.3%) over 18 years old. There was no statistical difference according to EBV detection frequency related to patient age among all the techniques studied ($p > 0.05$).

Positive cells were labeled in brown (Probes A and E), purple (Probes B, C and D). All probes used for ISH-EBER detected the EBV (**Figure**).

Thirty samples were hybridized with five different probes and analyzed by three pathologists, totalizing 450 results. In summary:

- 30 samples stained by one Probe analyzed by three pathologists = 90 results;
- 30 samples stained by five probes = 150 stained slides;
- 150 stained slides analyzed by three pathologists = 450 results.

Comparing the results, among the probes (**Table 2**) there was 46.7%, 42.2%, 50%, 30%, and 67.8% positive samples, respectively for Probe A, B, C, D and E. Probe E showed the highest proportion by Allred Score (2 to 8)[18].

Table 3 shows the evaluation of ISH regarding the quality; including comfort for evaluation, signal quality and homogeneity of labeled cell; Probe C followed by Probes A and E were better assessed by the pathologists. These probes also showed the best rates to morphological characteristics of the cells (**Table 4**), Probe E followed by Probes A and C. Probes C and D showed the lowest frequency of background (**Table 5**) and the Probes E and C are the ones with less artifacts (**Table 6**). The staining of cells, other than the R-S, was identified more frequently with Probe E (**Table 7**).

TABLE 2 – Evaluation of ISH labeled cells using Allred Score

Score	Probe A		Probe B		Probe C		Probe D		Probe E	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%
0	48	53.3	52	57.8	45	50	63	70	29	32.2
2-8	42	46.7	38	42.2	45	50	27	30	61	67.8
Total	90	100	90	100	90	100	90	100	90	100

Allred Score is the sum of intensity (0-1-2-3) and proportionality of labeled cells (0-1-2-3-4-5). Negative is 0 and positive ranges from 2 to 8. Probe E showed the highest % of labeled cells.

ISH: in situ *hybridization;* n*: indicates number of the results.*

TABLE 3 – Evaluation of ISH: reaction quality

Reaction quality	Probe A		Probe B		Probe C		Probe D		Probe E	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%
3	9	21.4	9	23.7	5	11.1	9	33.3	19	31.1
4	1	2.4	11	28.9	2	4.4	9	33.3	6	9.8
5	6	14.3	9	23.7	8	17.8	7	26	7	11.5
6	26	61.9	9	23.7	30	66.8	2	7.4	29	47.6
Total	42	100	38	100	45	100	27	100	64	100

Reaction quality included comfort for evaluation, signal quality and homogeneity of labeled cells. It was performed only in samples with positive results. All three parameters were evaluated as 1: bad and 2: good and the sum scored from 3 to 6. Probe C showed the best result.

ISH: in situ *hybridization;* n*: indicates number of the cases.*

TABLE 4 – Evaluation of ISH: morphology

Morphology	Probe A		Probe B		Probe C		Probe D		Probe E	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%
0	0	0	1	1.1	2	2.2	6	6.7	3	3.3
1	6	6.7	9	10	5	5.6	67	74.4	4	4.5
2	30	33.3	44	48.9	35	38.9	8	8.9	19	21.1
3	54	60	36	40	48	53.3	9	10	64	71.1
Total	90	100	90	100	90	100	90	100	90	100

Morphology was evaluated as 0: unanalysable; 1: no cell recognition; 2: difficult cell recognition; 3: good cell recognition. Probe E showed the best result.

ISH: in situ *hybridization;* n*: indicates number of the cases.*

TABLE 5 – Evaluation of ISH: background

Background reaction	Probe A		Probe B		Probe C		Probe D		Probe E	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%
0	77	85.6	62	68.9	83	92.2	82	91.1	69	76.7
1	13	14.4	28	31.1	7	7.8	8	8.9	21	23.3
Total	90	100	90	100	90	100	90	100	90	100

Background was evaluated as 0: absent; 1: present. Probe C showed the best results.

ISH: in situ *hybridization;* n*: indicates number of the cases.*

TABLE 6 – Evaluation of ISH: artifacts

Artifacts	Probe A		Probe B		Probe C		Probe D		Probe E	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%
0	63	70	63	70	73	81.4	69	76.7	76	84.4
1	27	30	27	30	17	18.2	21	23.3	14	15.6
Total	90	100	90	100	90	100	90	100	90	100

Artifacts was evaluated as 0: absent; 1: present. Probe E showed the best results.

ISH: in situ *hybridization;* n*: indicates number of the cases.*

TABLE 7 – Evaluation of ISH: labeling in cells other than Reed-Sternberg

Others cells	Probe A		Probe B		Probe C		Probe D		Probe E	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%
0	74	82.2	81	90	81	90	83	92.2	62	68.9
1	16	17.8	9	10	9	10	7	7.8	28	31.1
Total	90	100	90	100	90	100	90	100	90	100

Labeling in cells other than Reed-Sternberg was evaluated as 0: absent; 1: present. Probe D showed the best results.

ISH: in situ *hybridization;* n*: indicates number of the cases.*

The ISH-EBER results were compared to the IHC-LMP1 and PCR-EBNA1. The detection with IHC-LMP1 was 26.7% (8/30) and 66.7% (20/30) with PCR-EBNA1. **Table 8** shows the comparison of the EBV-positive rate using the three different detection methods, considering consensus the ISH-EBER results for the pathologists' analysis. Probe E and PCR showed agreement in results, both 66.7%.

Probes E and C presented the best values of agreement response, positive and also negative, among the three evaluators (**Table 9**). Probe E presented the best accuracy, sensitivity and specificity as compared to 100% of PCR (**Table 10**). It also showed the highest Cohen's Kappa index for the consensus evaluation (positive or negative) between the evaluators for the EBV detection in relation to PCR (**Table 11**).

Table 12 presents the average execution time and cost of the five probes, commercially available in São Paulo, Brazil. Probes A, B and E showed the lowest running time and Probe E was the one with the lowest cost. Considering all the results, Probe E is the one with the best cost benefit.

Regarding the test for probe specificity, Probe D labeled cells for CMV and HSV in the assessment of one pathologist. No positivity was observed for HHV8.

TABLE 8 – Comparison of the EBV-positive rate using different detection methods, considering ISH-EBER consensus results for pathologists analyses

Methods	Positive cases/*n*	%
IHC – LMP1	8/30	26.7
ISH – Probe A	13/30	43.3
ISH – Probe B	12/30	40
ISH – Probe C	15/30	50
ISH – Probe D	9/30	30
ISH – Probe E	20/30	66.7
PCR – EBNA1	20/30	66.7

EBV: Epstein-Barr virus; ISH: in situ *hybridization; EBER: Epstein-Barr encoded; IHC: immunohistochemistry; LMP1: latent membrane protein type 1; PCR polymerase chain reaction; EBNA1: Epstein Barr virus nuclear antigen 1;* n*: indicates number of the results.*

TABLE 9 – Evaluation of the concordance of responses (positive or negative) of ISH-EBER among evaluators, using each of the five probes

Probes	Fleiss' Kappa coefficient	*z*-score	*p*-value	Interpretation
Probe A	0.777	7.37	< 0.0001	Good
Probe B	0.636	6.03	< 0.0001	Good
Probe C	0.822	7.8	< 0.0001	Very good
Probe D	0.788	7.48	< 0.0001	Good
Probe E	0.900	8.54	0	Very good

Probe E showed the best response agreement value, positive and also negative, among the three evaluators.

ISH: in situ *hybridization; EBER: Epstein-Barr encoded.*

TABLE 10 – Evaluation of sensitivity, specificity, positive and negative predictive value, false-positive rate and accuracy of IHC and ISH with the five probes compared to PCR

Parameter	ISH					
	IHC	Probe A	Probe B	Probe C	Probe D	Probe E
Sensitivity	40	65	60	75	45	100
Specificity	100	100	100	100	100	100
PPV	100	100	100	100	100	100
NPV	45.5	58.8	55.6	66.7	33.3	100
False positive rate	0	0	0	0	0	0
Accuracy	60	76.7	73.3	83.3	63.3	100

Probe E presented the best sensitivity, specificity and accuracy.

IHC: immunohistochemistry; ISH: in situ *hybridization; PCR: polymerase chain reaction; PPV: positive predictive value; NPV: negative predictive value.*

TABLE 11 – Evaluation of matching replies (positive or negative) of the IHC and ISH of each of five probes, for the Nested-PCR results

	Cohen's Kappa coefficient	*z*-score	*p*-value	Interpretation
IHC	0.308	2.34	0.0195	Regular
Probe A	0.553	3.39	0.00070	Moderate
Probe B	0.500	3.16	0.00157	Moderate
Probe C	0.667	3.87	0.000108	Good
Probe D	0.353	2.54	0.0112	Regular
Probe E	1	5.48	< 0.0001	Very good

Probe E showed the highest Cohen's Kappa index.

IHC: indicates immunohistochemistry; ISH: in situ *hybridization; PCR: polymerase chain reaction.*

TABLE 12 – Average execution time and cost of the five probes, commercially available in São Paulo, Brazil

Probes	Average execution time	Cost – April/2012 US$
Probe A	5h45min	2572
Probe B	6h39min	1685
Probe C	26h37min	2478
Probe D	25h57min	1974
Probe E	6h43min	826

DISCUSSION

Detection of EBV may aid in the diagnosis of diseases related to viruses and influence the treatment and prognosis of the affected patients, thus, the accuracy of the detection is important. **Table 13** summarizes the references using some of the same probes of the current study. Note that some reports combined results of ISH-EBER and IHC-LMP1 which prevents meaningful comparative analysis. The integrity of the DNA or RNA is fundamental for ISH and it is known that the fixation, postfixation processing and preparation of sections affect the results[26]. The difference in results among studies using the same probe might be due the preservation of RNA, fixation, artifact or sample preparation.

TABLE 13 – References using the same probes of current study

References	Probes	Results	Notes
Pinto *et al*.[19]	Probe B	16/23 (69.5%)	
Huang *et al*.[20]	Probe B	126/491 (25.7%)	
Di Napoli *et al*.[21]	Probe B	10/16 (62.5%)	ISH-EBER + IHC-LMP1
Palma *et al*.[22]	Probe B	15/33 (45.5%)	ISH-EBER + IHC-LMP1
Tornóczky *et al*.[12]	Probe C	0/1 (0%)	
Vassallo *et al*.[23]	Probe C	36/61 (59%)	
Kwon *et al*.[24]	Probe C	8/28 (28.5%)	
Abd El All[25]	Probe C	6/10 (60%)	
Dinand *et al*.[4]	Probe D	32/33 (97%)	ISH-EBER + IHC-LMP1

ISH: in situ *hybridization; EBER: Epstein-Barr encoded; IHC: immunohistochemistry; LMP1: latent membrane protein type 1.*

Lauritzen *et al*. (1994)[1] utilized probe Y017 by Dako, a different probe from Probe B and reported positivity in 13/33 (39.4%), result similar to this study observed in Table 8 (40%).

Among the works carried out in Brazilian population Araújo *et al*. (2006)[27], Armstrong *et al*. (1993)[28], Elqui de Oliveira *et al*. (2002)[29], Souza *et al*.(2010)[30], Barros *et al*. (2010)[31] and (2011)[32] used probes different from those in the current study, in populations from the States of Bahia, São Paulo, Ceará and Rio de Janeiro. All six studies combined results of ISH-EBER and IHC-LMP1 preventing a comparative analysis.

Qi *et al*. (1998)[12] compared three techniques for EBV detection, PCR, IHC and ISH. Their results showed that the PCR was the most sensitive, but concluded that to find the location of infected cells in tissue, IHC and ISH are better.

Glaser *et al*. (2004)[33] compared ISH (EBER) and IHC (LMP-1) inter- and intra-observer reliability of EBV detection in HL and reported that EBER inter-observer agreement was the highest and LMP1 was the lowest for HLNS. In the current study, ISH results were higher than IHC, with a sensitivity of 100%, corroborating that the first was more reliable than the second one.

All five probes showed positivity, however, Probe E had the highest index (67.8%), improved sensitivity, specificity and accuracy (100%). It also showed a very good correlation between the different observers and the PCR and the better cost-benefit. The drawback of Probe E is that it showed the highest labeling in cells other than R-S. Some studies also described EBER detected in scattered small lymphocytes using Probe D[4], Biogenex probe[34] and other probes[1, 28, 35]. Weinreb *et al*. (1996)[36] considered EBV positivity, within others cells than R-S, may reflect the great sensitivity of ISH, and Gulley *et al*. (2002)[37] interpreted small EBER positive cells as latently infected lymphocytes that might be present in any viral carrier. Weiss *et al*. (1991)[35] compared ISH to PCR and found 9/12 (75%) positivity in others cells

than R-S, which PCR were positive for EBV-DNA in three/nine cases. All three cases showed the highest numbers of positive cells others than R-S by ISH, thus PCR positive results must be interpreted carefully.

EBV belongs to the subfamily of the gamma human herpesvirus, thus the evaluation of the specificity of the probes was performed by including samples confirmed by IHC. Probe D was the only one that stained few positive cells for CMV and HSV cases and showed the lowest rates of positivity and acceptance.

This current study suggests that two different detection methods should be performed, when available, to make the results more reliable, specially because EBV may serve as a target for therapeutic treatments[38], in the development of immunospecific therapies, epigenetic for cancers and may increase the rate of cure and for this purpose, a reliable diagnostic results are necessary.

CONCLUSION

The aim of this study was to evaluate the accuracy of EBV detection by ISH, using five different probes, commercially available, in São Paulo, Brazil, in cases of HLNS, and compare it to the results of IHC and PCR. The ISH-EBER results were compared to the IHC-LMP1 and PCR-EBNA1. The detection by IHC-LMP1 was 26.7% (8/30) and 66.7% (20/30) by PCR-EBNA1 and Probe E. For our laboratory conditions, Probe E showed the best results, demonstrating their applicability in the Laboratory of Public Health.

RESUMO

Introdução: *O vírus Epstein-Barr (EBV) pode servir como alvo nos tratamentos terapêuticos, sendo necessário resultado diagnóstico confiável.* ***Objetivo:*** *Avaliar a acurácia da detecção do EBV pela hibridização* in situ *(ISH), utilizando cinco sondas comerciais em amostras fixadas em formalina e incluídas em parafina de linfoma de Hodgkin (LH) esclerose nodular, comparando os resultados com a imuno-histoquímica (IHQ) e a reação em cadeia pela polimerase (PCR).* ***Material e método:*** *Trinta amostras foram selecionadas, sendo 28 linfonodos, uma medula óssea e um mediastino. Os seguintes parâmetros foram analisados: intensidade do sinal; proporcionalidade das células positivas; qualidade da reação de acordo com o conforto na avaliação, qualidade do sinal e homogeneidade das células marcadas; reação de fundo; morfologia; presença de artefatos; e positividade em outras células não neoplásicas. Todas as amostras foram analisadas para a detecção do EBV usando as cinco sondas, IHQ para proteína da membrana latente tipo 1 (LMP1) e PCR para antígeno nuclear do EBV (EBNA1). As análises estatísticas foram realizadas com o* software *R1; os índices de 5% para Kappa de Fleiss e Cohen foram considerados significantes.* ***Resultados:*** *A detecção pela IHQ-LMP1 foi de 26,7% (8/30) e 66,7% (20/30) pela PCR-EBNA1. Todas as sondas detectaram EBV. A positividade foi observada em 42/90 (46,7%), 38/90 (42,2%), 45/90 (50%), 27/90 (30%) e 61/90 (67,8%) para as sondas A, B, C, D e E, respectivamente.* ***Discussão:*** *Todas as sondas demonstraram positividade.* ***Conclusão:*** *A sonda E mostrou melhor taxa (67,8%), sensibilidade, especificidade e precisão (100%), boa correlação entre os diferentes observadores e com a PCR, além de ótimo custo/benefício.*

Unitermos: *infecções pelo vírus Epstein-Barr; hibridização* in situ*; doença de Hodgkin.*

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CORRESPONDING AUTHOR

Suely Nonogaki

Núcleo de Patologia Quantitativa; Centro de Patologia; Instituto Adolfo Lutz; Avenida Doutor Arnaldo, 355, 7º andar; Pacaembu; CEP: 01246-000; São Paulo-SP, Brasil; Phone: +55 (11) 3068-2874; e-mail: nonogaki@terra.com.br.