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Validation of chromogenic *in situ* hybridization reactions for DNA and RNA detection in formalin-fixed paraffin-embedded tissue

Validação das reações de hibridização in situ *cromogênica para detecção de DNA e RNA em tecidos fixados em formalina e incluídos em parafina*

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ABSTRACT

Introduction: Chromogenic *in situ* hybridization (CISH) is used alternatively to the traditional immunohistochemical methods for the diagnosis of infectious diseases in formalin-fixed paraffin-embedded samples, since it presents high sensitivity and specificity. This type of sample undergoes several chemical modifications during histological processing, and both poor and excessive fixation can impair sample quality, making it difficult to obtain good results. In CISH, it is common to use positive samples as quality control for the reactions; however, this practice does not provide any information regarding the preservation of the genetic material, nor does it avoid false-negative results. **Objective:** The objective of this study was to validate the deoxyribonucleic acid (DNA) (+) and (-), and ribonucleic acid (RNA) (+) and (-) control probes to be used as quality control for the samples, evaluating preservation of the genetic material. **Materials and methods:** Twelve histological sections were used (in quadruplicate, $n = 48$), prepared from a pool of tissues without microscopic changes related to infectious and/or inflammatory processes. The CISH protocol was conducted according to the manufacturer's instructions, standardized under the conditions of our laboratory, using commercial DNA and RNA probes chemically linked to digoxigenin. **Results and conclusion:** Our results were very satisfactory, showing high reproducibility, accuracy, sensitivity and analytical specificity, high predictive values for positive and negative assays and with zero ratio of false-positive and false-negative results, allowing the validation of this reaction.

Key words: *in situ* hybridization; quality control; molecular diagnostic techniques.

RESUMO

Introdução: *A hibridização* in situ *cromogênica (CISH) é uma alternativa aos métodos tradicionais imuno-histoquímicos para diagnóstico de doenças infecciosas em amostras fixadas em formalina e incluídas em parafina, visto que apresenta grande sensibilidade e especificidade. Esse tipo de amostra sofre diversas modificações químicas durante o processamento histológico, e tanto a má fixação quanto a fixação em excesso podem prejudicar a qualidade das amostras, inviabilizando bons resultados. Na CISH, é comum a utilização de amostras positivas como controle de qualidade das reações; entretanto essa prática não fornece nenhuma informação a respeito da preservação do material genético, nem evita resultados falso-negativos nas amostras testadas.* ***Objetivo:*** *O objetivo deste estudo foi realizar a validação das sondas comerciais para ácido desoxirribonucleico (DNA) (+) e (-) e ácido ribonucleico (RNA) (+) e (-), para serem utilizadas como controle de qualidade, avaliando a preservação do material genético nas amostras testadas.* ***Materiais e métodos:*** *Foram utilizados 12 cortes histológicos (em quadruplicata,* n = *48), confeccionados a partir de um* pool *de tecidos sem alterações microscópicas relacionadas com processos infecciosos e/ou inflamatórios. O protocolo de CISH foi conduzido de acordo com as instruções do fabricante e padronizado conforme as condições do nosso laboratório, utilizando sondas comerciais de DNA e RNA quimicamente ligadas à digoxigenina.* ***Resultados e conclusão:*** *Nossos resultados foram muito satisfatórios, demonstrando alta reprodutibilidade, acurácia, sensibilidade e especificidade analítica, bem como*

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altos valores preditivos para ensaios positivos e negativos e com proporção nula de resultados falso-negativos e falso-positivos, o que possibilitou a validação dessa reação.

Unitermos: *hibridização* in situ*; controle de qualidade; patologia molecular.*

RESUMEN

Introducción: *La hibridación in situ cromogénica (CISH) es una alternativa a los métodos tradicionales inmunohistoquímicos para el diagnóstico de enfermedades infecciosas en muestras fijadas en formol y embebidas en parafina, puesto que tiene alta sensibilidad y especificidad. Este tipo de muestra sufre diversas modificaciones químicas durante el procesamiento histológico, y tanto la mala fijación cuanto la fijación excesiva pueden perjudicar la calidad de las muestras, impidiendo buenos resultados. En la CISH, es común el empleo de muestras positivas para control de calidad de reacciones; sin embargo, esta práctica no proporciona ninguna información acerca de la preservación del material genético.* ***Objetivo:*** *El propósito de este estudio ha sido realizar la validación de las sondas comerciales para ácido desoxirribonucleico (ADN) (+) y (-) y ácido ribonucleico (ARN) (+) y (-), para que sean utilizadas como control de calidad, evaluando la preservación del material genético en las muestras testadas.* ***Material y métodos:*** *Se incluyen en el estudio 12 cortes histológicos (en cuadruplicado,* n = *48), confeccionados a partir de un pool de tejidos sin alteraciones microscópicas relacionadas con procesos infecciosos y/o inflamatorios. El protocolo de CISH se desarrolló de acuerdo a las instrucciones del fabricante y bajo las condiciones del nuestro laboratorio, haciendo uso de sondas comerciales de ADN y ARN quimicamente ligadas a digoxigenina.* ***Resultados y conclusión:*** *Nuestros resultados han sido muy satisfactorios, demostrando alta reproducibilidad, exactitud, sensibilidad, y especificidad analítica, así como altos valores predictivos para ensayos positivos y negativos, y con proporción nula de falsos negativos y falsos positivos, lo que ha permitido la validación de esa reacción.*

Palabras clave: *hibridación* in situ*; control de calidad; patología molecular.*

INTRODUCTION

Chromogenic *in situ* hybridization (CISH) is able to accurately localize specific nucleic acid sequences within fixed histological sections by binding to a complementary deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) sequence coupled to a reporter molecule. This methodological tool enables the acquisition of temporal and spatial information on expression and gene loci, based on steps in which a probe is synthesized, labeled, purified and hybridized to the specific target sequence. The great advantage in relation to the obtained results is the amount of information acquired by visualizing them results directly in the tissue. CISH in biopsy tissue has become a routine procedure in laboratories of pathological anatomy: it is applied in the study of structure, expression and gene characterization[1-6]. In this context, the Quantitative Pathology Center of the Pathology Branch of Instituto Adolfo Lutz (IAL) is responsible for conducting biomolecular tests for the diagnosis of diseases of public health significance (State Decree no. 55.601/2010) and currently uses CISH as a tool for the study of infectious diseases such as those caused by Epstein-Barr, cytomegalovirus and human papillomavirus. Formalin-fixed paraffin-embedded tissues undergo various chemical modifications during histological processing, and both poor and excessive fixation may impair sample quality and overall results for biomolecular studies[7]. In CISH, it is common to use positive samples as quality control indicators of the reactions; however, they are not able to assess sample quality, reflected by preservation of the genetic material, in order to avoid false-negative results. Therefore, the objective of this study was to validate the CISH technique for the DNA (+), DNA (-), RNA (+) and RNA (-) control probes to be used as quality control, in order to analyze the genetic material preservation of the samples.

MATERIALS AND METHODS

Samples

For this study, 12 histological sections were used (in quadruplicate, total $n = 48$) of 3 µm on silanized histological

slides, obtained from a pool of tissues (brain, spleen, lung and liver) without microscopic changes related to infectious and/or inflammatory processes, fixed in 10% buffered formalin. These were spare samples, which were processed for anatomopathological routine, included in this study after the end of custody of specimens according to temporality criteria established by the Institute. The fact they originated from different deceased patients, justified the waiver of informed consent [(CTC) IAL 11J-2017; CEP 2.439.900)].

Probes

Positive digoxigenin-linked probes were used for DNA detection (Zytovision, Bremerhaven, Germany – Ref. T-1022-100), which targets the human Alu repetitive sequences, and for RNA detection (Zytovision, Bremerhaven, Germany – Ref. T-1020-100), whose target is the 28S ribosomal RNA. Also, negative DNA (Zytovision, Bremerhaven, Germany – Ref. T-1023-100) and RNA (Zytovision, Bremerhaven, Germany – Ref. T-1119-400) probes, which contain a sequence of oligonucleotides with 40%-70% guanine-cytosine (GC), with no consensus for any naturally occurring sequence, were used to assess the presence of nonspecific bindings, which would cause background staining.

CISH kit

For this standardization, the kit used was the Zytofast Plus CISH Implementation kit HRP-DAB (Zytovision, Bremerhaven, Germany – Ref. T-1063-40).

Pre-hybridization

The histological sections were dewaxed in xylol and hydrated in ethanol. Endogenous peroxidase blocking was performed with a 6% hydrogen peroxide solution (Quimesp Química, Guarulhos, Brazil) and methanol (Merck, Darmstadt, Germany) (v/v) for 10 min, followed by enzymatic digestion by pepsin in a wet chamber at 37°C for 10 min, and pre-treatment by immersion in the Heat Pretreatment Solution at 95°C for 15 min, in a water bath.

Denaturation and hybridization

Positive (+) and negative (-) 10 µl probes for RNA and DNA were applied to the sections, which were covered with sealed coverslips. Denaturation occurred at 75°C for 5 min, and hybridization at 37°C for 21 h in the automatic hybridizer (Dako, Glostrup, Denmark).

Detection and visualization

After the hybridization period, the glue and coverslip were carefully removed and the slides were washed in Tris-buffered saline (TBS). After washing, we applied 20 µl of mouse anti-digoxigenin antibody to each slide and incubated them at 37°C for 60 min in a humid chamber. Three washings were then performed in Wash Buffer TBS, which preceded the application of 20 µl of the anti-mouse antibody, bound to a polymer with horseradish peroxidase (HRP), and the slides were incubated at 37°C for 90 min in a humid chamber. We proceeded with three washes in Wash Buffer TBS and development with 50 µl of diaminobenzidine (DAB) solution. The slides were incubated at 37°C for 10 min, washed in distilled water and counterstained with hematoxylin.

Standardization of the analysis method

After the reaction was complete, the slides were analyzed by three pathologists and one analyst. The criterion of positivity was the visualization of a brown precipitate in the nucleus for the labeling of DNA, and in the nucleus and cytoplasm of the tissue cells for the labeling of RNA. Samples that were described as not showing brownish precipitation in cellular components by one of the professionals were considered negative.

For analysis purposes, the reactions were divided into two groups according to the target (DNA or RNA), which were analyzed separately. In turn, these groups were subdivided into positive and negative samples, according to the probe used.

Intermediate accuracy (intra-assay) or repeatability

The reactions were performed on different days, and to calculate the intermediate accuracy (repeatability), we used only the descriptive analysis of the observed results.

Analytical sensitivity (Se [%])

To evaluate the ability of the reaction to detect the specific nucleic acid sequence, when using a positive probe, the following formula was used: $Se = TP/(TP + FN)$, where TP (true positive) is the number of positive results in samples where positive probes were used, and FN (false-negative) is the number of negative results in samples where positive probes were used.

Analytical specificity (Sp [%])

To evaluate the ability of the reaction not to detect the specific nucleic acid sequence, when using a negative probe, the following formula was used: $Sp = TN/(TN + FP)$, where TN (true negative)

is the number of negative results in samples where negative probes were used and FP (false-positive) is the number of positive results in samples where negative probes were used.

Predictive value study for a positive test result (PPV [%])

In order to study the probability that a sample with positive CISH for certain nucleic acid actually have the presence of the nucleic acid, the following formula was used: positive predictive value (PPV) = TP/(TP + FP), where TP is the number of positive results in samples where positive probes were used and FP is the number of positive results in samples where negative probes were used. For the calculation of the proportion of false-positives, the following formula was used: (pFP) = 1 - specificity.

Predictive value study for a negative test result (NPV [%])

In order to study the probability of a sample with negative CISH for certain nucleic acid not really have the presence of the nucleic acid, the following formula was used: negative predictive value (NPV) = TN/(TN + FN), where TN is the number of negative results in samples where negative probes were used and FN is the number of negative results in samples where positive probes were used. For the calculation of false-negative ratio, the following formula was used: (pFN) = 1 - sensitivity.

Accuracy (A [%])

In order to evaluate the degree of correspondence between the results obtained by CISH in relation to the expected results, i.e. positive in the presence of the positive and negative probes in the presence of the negative probe for nucleic acids, the following formula was used: A = (TP + TN)/N.

RESULTS

In all slides where the positive DNA (**Figure 1**) or RNA (**Figure 2**) probes were applied, it was possible to observe a brown precipitate in the different histological sections. In contrast, in the slides where DNA or RNA negative probes were applied, it was not possible to observe any precipitate or background staining, indicating absence of cross reactions.

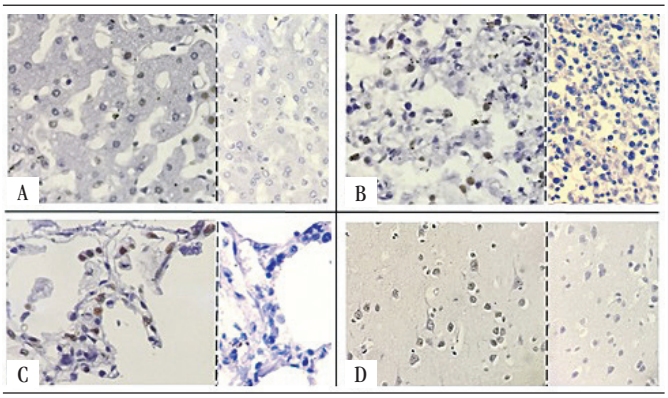

FIGURE 1 – *CISH reactions for DNA detection. Photomicrography of the CISH reaction for DNA*
A) liver; B) spleen; C) lung; D) brain; magnification 40×. It is possible to observe the brown precipitate in the cell nucleus resulting from the enzymatic reaction of the DAB with the peroxidase of the antibody complex bound to the hybrid formed between the target sequence and the probe in the reactions with the positive (right) probe in comparison to the reactions with the negative probe (left).

CISH: chromogenic in situ *hybridization; DNA: deoxyribonucleic acid; DAB: diaminobenzidine.*

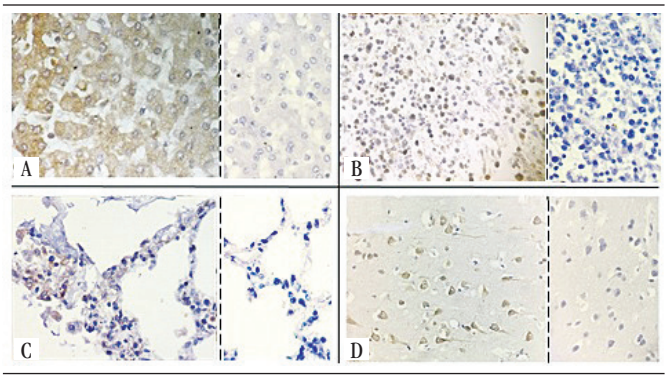

FIGURE 2 – *ISH reactions for RNA detection. Photomicrography of the CISH reaction for ribosomal RNA 28S*
A) liver; B) spleen; C) lung; D) brain; magnification 40×. It is possible to observe the brown precipitate in the nucleus and cellular cytoplasm resulting from the enzymatic reaction of the DAB with the peroxidase of the complex of antibodies bound to the hybrid formed between the target sequence and the probe in the reactions with the positive (right) probe in comparison to the reactions with the negative probe (left).

ISH: in situ *hybridization; CISH: chromogenic* in situ *hybridization; RNA: ribonucleic acid; DAB: diaminobenzidine.*

The results obtained for each probe are described in **Table**. For both DNA and RNA probes, positive and negative, it is possible to observe that there was 100% (n = 24) agreement of the results between the different days of the reactions, demonstrating the appropriate repeatability of the method.

The results of the CISH reactions for both nucleic acids demonstrated 100% sensitivity, analytical specificity and accuracy. Predictive values for positive and negative assays were also 100%, while the proportion of false-negative and false-positive assays was zero.

TABLE – Chromogenic *in situ* hybridization reactions for DNA and RNA detection paired with probes

		Results			
		DNA (+)	DNA (-)	RNA (+)	RNA (-)
Probes	DNA (+)	12	0	-	-
	DNA (-)	0	12	-	-
	RNA (+)	-	-	12	0
	RNA (-)	-	-	0	12
	Total	12	12	12	12

From these results, we calculated the analytical sensitivity and specificity, predictive value for a positive and negative test result, the proportion of false-negative and false-positive results, and accuracy.

DNA: deoxyribonucleic acid; RNA: ribonucleic acid; +: positive results, -: negative results.

DISCUSSION

CISH allows the use of formalin-fixed paraffin-embedded tissues for individual gene monitoring in cells within the context of the observed tissue using DNA or RNA probes complementary to the sequences of interest[8-10]. Biomolecular techniques, such as CISH, are used alternatively to traditional immunohistochemical methods for the diagnosis of infectious diseases, since they present higher sensitivity and specificity[9, 10]. However, for this technique to be successful, it is necessary that the tissue has been properly fixed, allowing both the preservation of its genetic material and its cytoarchitecture. Formalin fixation leads to nucleic acid crosslinking to proteins and other cellular constituents, making it difficult to bind to complementary probes[11, 12]. Therefore, the recovery step, which precedes the binding with the probe, becomes very important since it allows the breaking of some of these bonds.

Several other factors also affect the quality of nucleic acids in paraffinized tissues, such as fixative pH, tissue fixation time, amount of time elapsed prior to sample fixation[13], as autolysis begins as soon as the sample is removed[14]. In addition, formalin-fixed paraffin-embedded tissue storage conditions and the method used for extraction should also be considered[13].

In the context of CISH assays, in routine laboratories, it is not always possible to have control over fixation of the tissue to be analyzed. Therefore, it is impossible to know if a negative result for a particular probe is due to the absence of the targeted sequence in the cells or due to tissue degradation, making it impossible to hybridize the probe. In the second case, poorly conserved genetic material may imply false-negative results, with great impact on patient diagnosis and clinical conduct. That is the reason why it is important to use control probes for DNA or RNA detection, depending on the primary target being aimed for. Therefore, our results demonstrated that positive and negative DNA and RNA probes can be employed in the routine as quality control for the samples, validating the negative results in all conditions that have CISH as a diagnostic tool.

CONCLUSION

Our results demonstrated that this protocol for CISH – DNA and RNA – had very satisfactory results with high reproducibility, accuracy, sensitivity and analytical specificity, high predictive values for positive and negative assays, with zero ratio of false--positive and false-negative results, enabling a standardization of this reaction for use in routine.

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