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Original Article

Assessment of Antigen Retrieval Solution in Immunohistochemistry

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Abstract

Immunohistochemistry (IHC) is a critical diagnostic tool that depends on antigen–antibody interactions to detect protein expression in formalin-fixed paraffin-embedded tissues. However, formalin fixation often masks antigenic sites, necessitating antigen retrieval to restore epitope accessibility. This study evaluated seven antigen retrieval solutions; hydrochloric acid, glacial acetic acid, formic acid, citric acid, oxalic acid, ethylene diamine tetra acetic acid, and water across three concentrations (0.1%, 0.2%, and 0.3%) using breast and appendix tissues. The Quick Score method, which combines staining intensity and the proportion of positive cells, was applied to assess the performance of each solution in detecting three antigen markers: Progesterone receptor (PR), CD20, and Pan-cytokeratin. Results demonstrated that citric acid consistently produced the highest Quick Scores at 0.1% and 0.3%, with a slight decrease in CD20 expression at 0.2%, indicating that minor concentration-dependent fluctuations may influence some antigens. Hydrochloric acid, glacial acetic acid, and formic acid at 0.1%, 0.2%, and 0.3% also showed strong retrieval efficiency, although responses varied across concentrations. In contrast, oxalic acid was highly effective for Pan-cytokeratin at 0.1%, 0.2%, and 0.3%, but performed poorly for PR and CD20 across concentrations, indicating antigen-specific retrieval capacity. Staining response varied when different retrieval solutions were used. The citric acid solution showed a relatively consistent staining pattern in the examination of PR, CD20, and Pan-cytokeratin compared with several other retrieval solutions. This finding indicates that the type of retrieval solution and its concentration need to be considered in the antigen retrieval process to obtain optimal immunohistochemical staining results.

Keywords: Antigen Retrieval Solutions, Formalin Fixed Paraffin Embedded Tissue, Immunohistochemical Staining.

1. Introduction

Immunohistochemistry (IHC) is a laboratory technique that uses antibodies to detect the protein and other antigens in the tissue sections. Antibodies are specific, would bind to the antigen of interest. The antibody-antigen interaction is then viewed using either a colored enzyme substrate (chromogenic detection) or a fluorescent dye in fluorescent detection (Gamde et al., 2024). Tissue processing for routine pathology and special techniques, such as IHC, includes fixation in formalin and embedding in the paraffin wax. This procedure denatures tissue proteins and may lead to masking of epitopes. Antigen retrieval is a method

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to re-expose hidden antigens in formalin-fixed and paraffin-embedded specimens, so that antigens can be detected by antibodies (Shi et al., 2011).

The antigen-masking effects of fixation are reversible to varying degrees by a process known as antigen retrieval. It involves exposing the tissue sections to heat or proteolytic enzyme digestion before performing immunohistochemical staining. The success of retrieval depends on the fixative, the duration of fixation, and the antigen of interest. Nowadays, immunohistochemical staining is routinely practiced in routine pathology. One of the problems in detecting antigens is the unmasking of these antigens in formalin-fixed, paraffin-embedded tissues through an antigen retrieval (AR) step (Tsutsumi, 2020). HIER is the more widely used technique that involves heating tissue sections to break the cross-links formed during fixation, thereby restoring the antigenic sites. This method can be performed using several appliances, including: microwave, ovens, pressure cookers, and water baths (ABCAM, 2025).

Although antigen retrieval is often used in IHC, there remains a disagreement on which retrieval solution is most effective for various antigens. Previous research and existing literature have primarily relied on the use of citric acid and EDTA. However, using higher pH and EDTA-based solutions has shown inconsistent, even conflicting results and limitations, including the loss of sections, tissue damage, loss of structural integrity, particularly, in delicate specimens. The existing antigen retrieval methods are not easy to standardize. It could produce different results within the same or different laboratories (Sompuram et al., 2015). For that reason, it is necessary to carefully and thoroughly develop alternative antigen retrieval solutions in immunohistochemistry applications and compare the existing techniques.

The use of various types of retrieval solutions in this research is based on the consideration that the antigen retrieval process is influenced not only by the type of solution used, but also by the chemical characteristics of the solution and its concentration. Acid-based solutions can affect the interaction between tissue proteins and antigenic epitopes through different chemical conditions, so that the antigen retrieval response can vary across each immunohistochemical marker. Therefore, the use of several solutions at different concentrations was employed to illustrate the variation in staining response for the PR, CD20, and Pan-cytokeratin markers.

This research aims to evaluate the immunohistochemical staining response using various antigen retrieval solutions at several concentrations toward the expression of PR, CD20, and Pan-cytokeratin in breast tissue and appendix tissue.

2. Literature Review

2.1. Immunohistochemistry and Antigen Masking

Immunohistochemistry (IHC) is an analytical technique used to identify and localize specific antigens within tissue sections through antigen-antibody interactions. The technique is widely applied in diagnostic pathology and biomedical research because it enables the detection of specific proteins while maintaining the morphological characteristics of tissue. In routine practice, tissue specimens are commonly fixed in formalin and embedded in paraffin to preserve tissue architecture. However, the fixation and processing procedures may alter the molecular characteristics of tissue proteins and consequently reduce the accessibility of specific antigenic epitopes to antibodies (D'Amico et al., 2009; Ramos-Vara, 2017).

Formalin fixation can produce cross-linking between protein molecules, resulting in masking or conformational changes of antigenic epitopes. This condition may interfere with the interaction between the target antigen and primary antibody and consequently produce

weak or undetectable immunohistochemical staining. Therefore, antigen retrieval is commonly incorporated into IHC protocols for formalin-fixed, paraffin-embedded (FFPE) tissue to improve the accessibility of masked epitopes and enhance antigen detection (Shi et al., 1997).

2.2. Antigen Retrieval in Immunohistochemistry

Antigen retrieval is a pretreatment procedure designed to restore or increase the accessibility of antigenic epitopes that have become masked during tissue fixation and processing. The development of antigen retrieval has substantially improved the detection of proteins in FFPE tissue and has expanded the application of IHC in diagnostic and research settings. Antigen retrieval can be broadly categorized into heat-induced epitope retrieval (HIER) and proteolytic-induced epitope retrieval (PIER). HIER uses heat in combination with an appropriate retrieval solution, whereas PIER uses proteolytic enzymes such as trypsin, pepsin, or proteinase K to modify the proteins surrounding the masked epitope (Krenacs et al., 2009; Shi et al., 2011).

HIER is particularly widely applied in routine IHC because it can improve antigen detection in formalin-fixed tissues while preserving tissue morphology when appropriately optimized. The effectiveness of HIER is influenced by several parameters, particularly the composition and pH of the retrieval solution, heating temperature, and duration of treatment. Excessive heating or prolonged treatment may cause tissue deterioration, whereas insufficient treatment may result in inadequate antigen retrieval. Consequently, antigen retrieval conditions need to be optimized according to the antigen-antibody combination and characteristics of the tissue specimen (Krenacs et al., 2009; Shi et al., 2011).

2.3. Antigen Retrieval Solutions and Assessment of Their Effectiveness

The retrieval solution is an important component of HIER because its chemical composition and pH influence the efficiency of epitope recovery. Commonly used retrieval solutions include citrate buffer at approximately pH 6.0, Tris-HCl at approximately pH 8.0, EDTA-based solutions at approximately pH 8.0, and Tris–EDTA solutions at approximately pH 9.0. These solutions may produce different levels of antigen recovery depending on the target antigen, tissue characteristics, fixation conditions, and heating protocol (Krenacs et al., 2009).

The effectiveness of an antigen retrieval solution can be assessed through the quality of immunohistochemical staining, including staining intensity, proportion of positive cells, specificity of staining, background staining, and preservation of tissue morphology. Previous studies have demonstrated that no single antigen retrieval protocol is universally optimal for all antigen–antibody combinations. Different retrieval solutions and pH conditions may therefore need to be evaluated to determine the condition that provides adequate antigen detection while minimizing nonspecific staining and tissue damage (Pileri et al., 1997).

A comparative study by Pileri et al. (1997), for example, evaluated antigen retrieval using citrate buffer pH 6.0, Tris-HCl pH 8.0, and EDTA-NaOH pH 8.0 across multiple antibodies and demonstrated that the performance of antigen retrieval varied according to the antibody and target antigen. These findings support the need for comparative assessment when selecting an appropriate retrieval solution for a particular IHC application.

3. Methods

3.1. Research Design

A semi-quantitative approach was used for the IHC staining. The results are expressed through grading system based on the staining intensity and proportion of the positive cells. Such scoring gives a reliable estimation of antigen expression that can be systematically compared across experimental setting (Detre et al., 1995).

To evaluate the effect of different antigen retrieval solutions, including citric acid buffer, EDTA, oxalic acid, hydrochloric acid, formic acid, glacial acetic acid buffer, and water across various antigen and tissue types using a controlled experimental design in heat-induced epitope retrieval (HIER), the solutions would be compared. Each AR solution was evaluated in standardized laboratory conditions and compared for the ability to improve antigen detection (Shi et al., 2011). The protocols were thoroughly examined in order to find patterns, trends, and conclusions about the relative efficacy of each antigen retrieval solution due to the research design. To draw insightful conclusions about the effectiveness of various antigen retrieval solutions, the results from these sources were then compared.

3.2. Study Area

The study was carried out in the histopathology laboratory of National Hospital Abuja, where preserved formalin-fixed paraffin-embedded (FFPE) tissue sections were processed, subjected to immunohistochemistry and examined.

3.3. Sample Size and Techniques

A total of 69 human tissue sections were used for this study. The samples for this study included routine sections of normal formalin-fixed, paraffin-embedded (FFPE) tissue samples taken from a histopathology laboratory. Two different types of tissues were utilized for each antigen retrieval solution: breast tissue and appendix tissue, to evaluate antigen retrieval solutions' effectiveness. Buffer solutions were prepared at concentrations of 0.1%, 0.2%, and 0.3% for each of the following acids: citric acid, EDTA, oxalic acid, hydrochloric acid, formic acid, glacial acetic acid, and water respectively.

The number of slides used in the research was determined based on the combination of the type of retrieval solution, solution concentration, and immunohistochemical marker as described in the research design. The calculation of the number of slides followed all combinations of treatments used in the research and included control slides.

3.4. Calculations

The total number of slides was determined based on the number of antigen retrieval solutions, concentrations, markers, and controls used in the study. There were 7 antigen retrieval solutions, each tested at 3 concentrations (0.1%, 0.2%, and 0.3%) using 3 markers (PR, CD20, and pan-cytokeratin):

7 antigen retrieval solutions × 3 concentrations × 3 markers = 63 slides

For each antigen retrieval solution:

A. 0.1%:PR, CD20, pan-ck → 3 slides
B. 0.2%:PR, CD20, pan-ck → 3 slides
C. 0.3%:PR, CD20, pan-ck→ 3 slides = 9 slides for each antigen retrieval solution

The study also included 2 controls (positive and negative controls) for each of the 3 markers:
Controls: (2 controls, positive & negative for 3 markers) = 6 slides
Therefore, the total number of slides was:
Total number of slides = 69 formalin-fixed paraffin embedded (FFPE) tissue samples.

For solution preparations, to prepare 100ml of 0.1%, 0.2%, and 0.3% of the reagents (Hydrochloric acid, glacial acetic acid, formic acid, citric acid, oxalic acid, EDTA).
The general dilution formula used: C1V1= C2V2
Where:
C1: Concentration of initial stock (1%) V1: volume of stock solution needed
C2: Final concentration
V2: Final volume (100ml)

A. HYDRO CHLORIC ACID SOLUTION PREPARATION:
   1) 0.1%: V1=0.1×100= 10ml of HCL + 90 ml distilled water 1
   2) 0.2%: V1=0.2×100= 20ml of HCL + 80 ml distilled water 1
   3) 0.3%: V1=0.3×100= 30ml of HCL + 70 ml distilled water 1

B. GLACIAL ACETIC ACID SOLUTION PREPARATION
   1) 0.1%: V1=0.1×100 = 10 ml of GA + 90 ml distilled water 1
   2) 0.2%: V1=0.2×100 = 20 ml of GA +80 ml of distilled water 1
   3) 0.3%: V1=0.3×100 = 30 ml of GA +70 ml of distilled water 1

C. FORMIC ACID SOLUTION PREPARATION:
   1) 0.1%: V1=0.1×100= 10ml of FA + 90 ml of distilled water 1
   2) 0.2%: V1=0.2×100= 20 ml of FA + 80 ml of distilled water 1
   3) 0.3%: V1=0.3×100= 30 ml of FA + 70ml of distilled water 1

D. CITRIC ACID SOLUTION PREPARTION:
   1) 0.1%: V1=0.1×100= 10 ml of CA + 90 ml of distilled water 1
   2) 0.2%: V1=0.2×100= 20 ml of CA + 80 ml of distilled water 1
   3) 0.3%: V1=0.3×100= 30 ml of CA + 70 ml of distilled water 1

E. OXALIC ACID SOLUTION PREPARATION:
   1) 0.1%: V1=0.1×100= 10 ml of OA + 90 ml of distilled water 1
   2) 0.2%: V1=0.2×100= 20 ml of OA + 80 ml of distilled water 1
   3) 0.3%: V1=0.3×100= 30 ml of OA + 70 ml of distilled water 1

F. EDTA (Ethylene diamine tetra acetic acid) SOLUTION PREPARATION:
   1) 0.1%: V1=0.1×100= 10 ml of EDTA + 90 ml of distilled water 1
   2) 0.2%: V1=0.2×100 = 20 ml of EDTA + 80 ml of distilled water 1
   3) 0.3%: V1=0.3×100= 30 ml of EDTA + 70 ml of distilled water 1

For the preparation of the three solutions of water (neutral, slightly acidic, and alkaline), distilled water was divided into three parts and adjusted according to different Ph levels. Plain distilled water was used to represent the neutral condition. To make the slightly acidic solution, 1 ml of concentrated hydrochloric acid (HCL) was added to 99 ml of distilled water in the first labeled Coplin jar and set aside and for the alkaline solution, 1ml of ammonia solution was added to the measured 99 ml of distilled water in the third labeled Coplin jar.

3.5. Materials

The following materials and solutions were appropriately utilized in this research work: glass slides, Xylene, water, coplin jars, Absolute alcohol, 90% alcohol, buffer solutions [hydrochloric acid, glacial acetic acid, formic acid, water, citric acid, oxalic acid, ethylene diamine tetra acetic acid (EDTA)], phosphate buffered saline (PBS), antigen markers (CD20, Pan-cytokeratin, Progesterone receptor; PR), protein block, horseradish peroxidase (HRP), avidin, chromogen substrate; Diaminobenzidine (DAB), hematoxylin, litmus Ph test strips, DPX mountant, cover slip. Equipment used: LEICA automatic tissue processing machine, tissue embedding machine, and rotary microtome, and pressure cooker.

3.6. Method for Data Collection

In relation to antigen retrieval (AR) and immunohistochemistry (IHC), a number of data collection techniques were used to compare the efficacy of various antigen retrieval solutions in IHC. However, the technique used in this study was the heat-induced AR, which is commonly used for archival formalin-fixed, paraffin-embedded (FFPE) tissue sections with the tissue undergoing cycles of pressure cooking or microwave treatment.

3.6.1. Tissue Sectioning and Processing

The data collection was carried out on grossed tissue sections that has undergone normal tissue processing procedures of fixing in 10 neutral buffered formalin, dehydration in series of alcohol; 70%, 80%, 90%, 95% to 100%, clearing to remove the dehydrant by replacing the alcohol with xylene, infiltrating using Automatic tissue processor and embedding of the tissues in paraffin wax to provide support for sectioning using embedding machine. The Paraffin blocks were prepared and sectioned at 3 µm thickness using a microtome as described by Amina et al. (2024) ready for antigen retrieval process. Before staining, slides were deparaffinized with xylene to remove paraffin for 2 minutes, the tissue was rehydrated using 2 changes of graded levels of alcohols and tissue section was taking to water for 1 minute.

3.6.2. Antigen Retrieval: Heat induced Antigen Retrieval

This method involved heating formalin-fixed, paraffin-embedded (FFPE) tissue sections in a retrieval solution and is frequently used for FFPE tissue sections. HIER involved heating the tissue section in a buffer of specific pH with any antigen retrieval solution at high temperature (Janardhan et al., 2018). The appropriate antigen retrieval buffer was added into 63 Coplin jars. The slide sections were labeled according to their solutions, markers, concentrations and each placed in their retrieval solutions.

This was followed by placing the Coplin jars into the pressure cooker and closing the lid, a timer was set to boil for 25 minutes. After boiling, the slides were allowed to cool in the buffer for further 5 minutes, rinsed in running tap water, rinsed with PBS, and drained. It is advised to conduct a control experiment to optimize retrieval time.

3.6.3. Antigen Retrieval Blocking/Protein Blocking

The slide section was then blocked by applying and treating with protein block for 10 minutes. The protein block was drained from the section and rinsed with PBS. Without this step, the primary antibody may bind to several sites and cause a false-positive result. Antibodies found in serum bind to non-specific sites, making it common protein-blocking agents, preventing non specific binding to the numerous hydrophobic side chains of the proteins found in the tissue. A blocking procedure is done during multiple IHC staining procedures to prevent cross-antigen/antibody reaction (Shi et al., 2001).

3.6.4. Primary Antibody Incubation

The slide sections were arranged on a slide incubation chamber, and the appropriate primary antibodies were applied on each slide section, incubated and covered for 1 hour in a humidity chamber to maintain a humid environment. After incubation, unbound primary antibody was washed away using PBS (phosphate-buffered saline). A primary antibody's optimal conditions may vary depending on the tissue type and experiment.

3.6.5. Secondary Antibody Incubation and Detection

After incubation of primary antibody, labeled secondary antibody (avidin) was applied for 20 minutes. Following incubation, the slides were thoroughly rinsed with PBS to get ready for any unbound antibodies. Indirect detection is used which involves incubating the tissue

sample with a secondary antibody that is conjugated to a visualization system and identifies the main antibody. For the detection, HRP (Horseradish peroxidase) was applied to the slide section for 30 minutes and rinsed with PBS. This was followed by application of chromogen substrate (DAB) which was prepared by mixing (2000µm of buffer substrate + 1 drop of chromogen) for 3 minutes ensuring to rinse the slide in PBS thoroughly after application. At the site of antigen binding, HRP, one of the most commonly used enzymes for chromogenic detection, converts substrates (DAB) into colorful brown precipitates (Seidal et al., 2001). IHC detection method primarily depend on a reagent typically an antibody that attaches to the primary antibody to enhance signal production and target antigen visualization. The antibody is detected indirectly using a labeled secondary antibody.

3.6.6. Counter Staining and Mounting

After the primary antibody was observed using HRP and DAB, the stained tissue slides were counter stained using hematoxylin for 2 minutes, blued in running tap water and rinse. Finally, the slide sections were dehydrated in grades of alcohol, cleared in xylene, mounted in DPX mountant and viewed under the microscope to access antigen expressions. The interpretation of immunostaining depends on the quantity of antigen present in the tissue (Renshaw, 2013).

3.6.7. Method of Assessment

Immunostaining was evaluated using a light microscope and the IHC stained slides were evaluated using a semi-quantitative method known as The Quick score, which considers both the proportion of stained cells and the intensity of the stain (Renshaw, 2013). The staining was assessed only on the invasive component and evaluated the sections with the best preservation and staining (Detre et al., 1995). The Quick score technique used to assess staining outcomes is expressed as follows.

Based on the intensity of staining in the cells

+1 (weak stain); +2 (moderate stain); +3 (strong stain).

Based on the proportion of labeled cells:

Score 1 indicates that<25% of the cells are stained.

Score 2: more than 25% of cells stained but not up to 50%.

Score 3: If up to 50% of the cells are stained.

Score 4 indicates that up to 75% of the cells are stained.

Score 5 indicates that more than 75% to 100% of the cells are stained.

The overall score is calculated by adding the scores for the proportion of stained cells and staining intensity 3+5= 8. We achieved the following scores: (2&3) low; (4&5) moderate; (6, 7, & 8) high. Therefore, the total outcomes varied from 0 to 8, with 0 considered as negative staining.

4. Results and Discussion

4.1. Research Results

Data from quick scores was collected and compared between AR solutions and concentration levels. The results were illustrated below using tables and bar charts and photographs which were assessed as percentages and staining intensity. The effectiveness of the different solutions was evaluated by comparing their quick score performance.

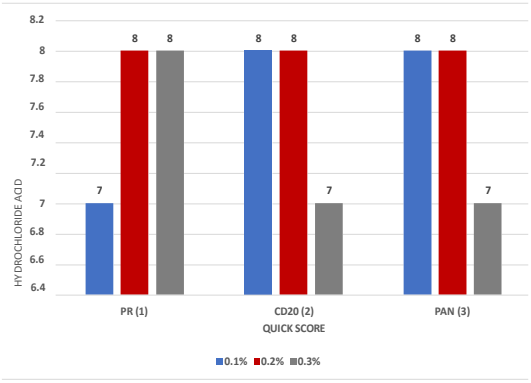


Figure 1. Hydrochloric Acid Concentration

Figure 1, showed progesterone (PR) improved with concentration: it was 7/8 at 0.1% and increased to 8/8 at both 0.2% and 0.3%. The CD_{20} marker showed the strongest results at both 0.1% and 0.2%, achieving 8/8, but slightly decreased to 7/8 at 0.3%. The Pan-Cytokeratin marker remained high at 0.1% and 0.2%, with a result of 8/8, but decreased to 7/8 at 0.3%. Representative photomicrographs of the immunohistochemical staining obtained with hydrochloric acid are presented in Figure 2.

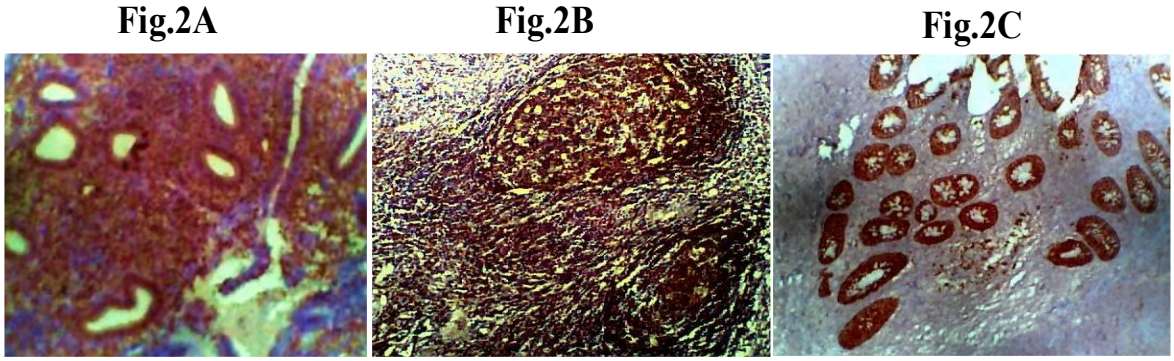


Figure 2. Immunohistochemical staining using hydrochloric acid (HCl) as the antigen retrieval solution across three concentrations. (A) Endometrial tissue stained with progesterone receptor (PR) marker using 0.1% HCl. (B) Appendix follicle tissue stained with CD20 marker using 0.2% HCl. (C) Appendix tissue stained with Pan-cytokeratin marker using 0.3% HCl. All images captured at 10x objective magnification

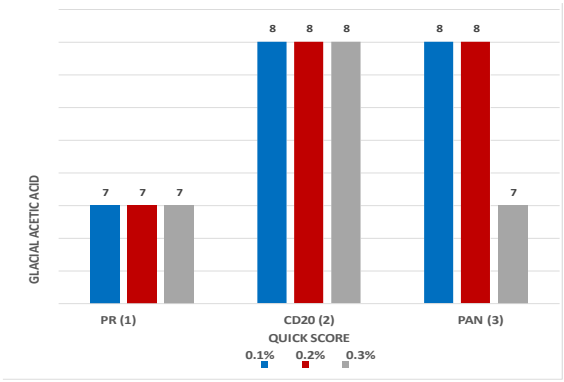


Figure 3. Glacial Acetic Acid Concentration

Figure 3, showed progesterone (PR). Glacial acetic acid remained consistent at 7/8 across all concentrations. CD20 showed consistent strong staining (8/8) across all concentrations. Pan -Cytokeratin was best between 0.1% and 0.2% (8/8), with a slight reduction at 0.3% (7/8). Representative photomicrographs of the immunohistochemical staining obtained with glacial acetic acid are presented in Figure 4.

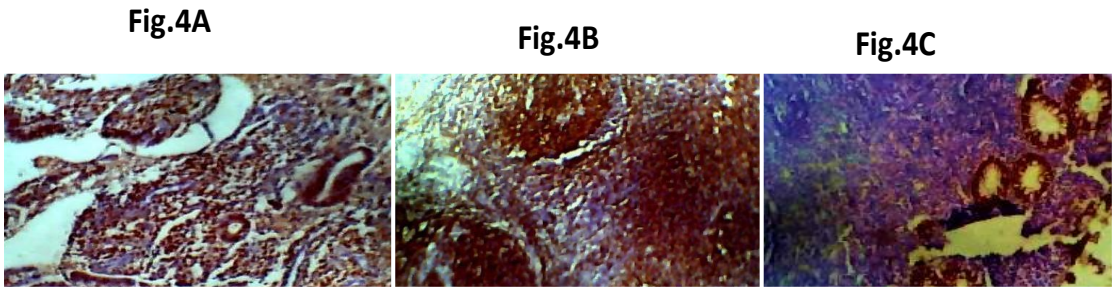


Figure 4. Immunohistochemical staining using glacial acetic acid as the antigen retrieval solution across three concentrations. (A) Endometrial tissue stained with progesterone receptor (PR) marker using 0.1% glacial acetic acid. (B) Appendix follicle tissue stained with CD20 marker using 0.2% glacial acetic acid. (C) Appendix tissue stained with Pan-cytokeratin marker using 0.3% glacial acetic acid. All images captured at 10x objective magnification.

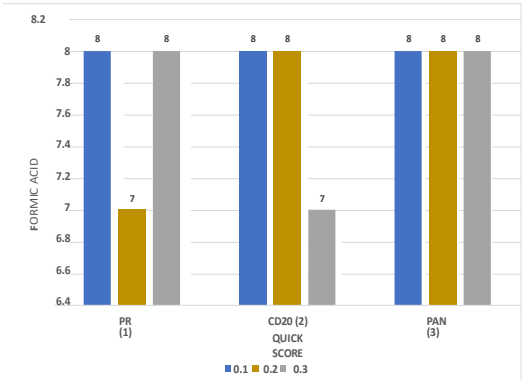


Figure 5. Formic Acid Concentration

Figure 5 shows excellent formic acid progesterone (PR) at 0.1% and 0.3% (8/8) result, but slightly lower at 0.2% (7/8). CD_{20} levels remained high at 0.1% and 0.2% (8/8), but decreased to 0.3% (7/8) while Pan-Cytokeratin maintained 8/8 at all concentrations and was very stable with formic acid retrieval. In summary, Formic acid at 0.1% provided excellent results (8/8 across all markers), making it the best-performing concentration for this solution. Representative photomicrographs of the immunohistochemical staining obtained with formic acid are presented in Figure 6.

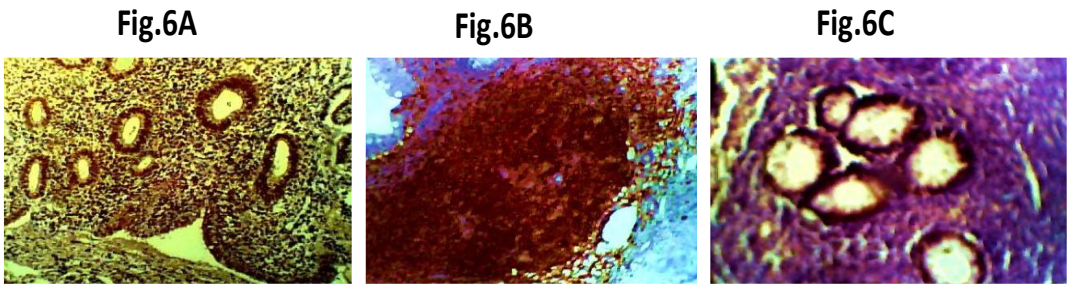


Figure 6. Immunohistochemical staining using formic acid as the antigen retrieval solution across three concentrations. (A) Endometrial tissue stained with progesterone receptor (PR) marker using 0.1% formic acid. (B) Appendix follicle tissue stained with CD20 marker using 0.2% formic acid. (C) Appendix tissue stained with Pan-cytokeratin marker using 0.3% formic acid. All images captured at 10x objective magnification

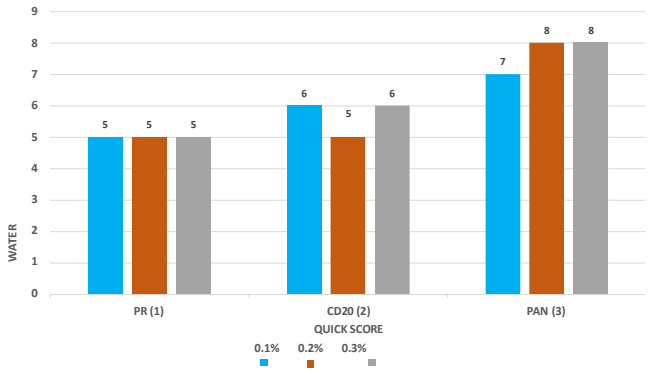


Figure 7. Water Concentration

Figure 7 showed progesterone (PR) is low in alkaline and neutral waters (5/8), but excellent in acidic waters (8/8). For CD_{20}, moderate in alkaline and acidic (6/8); slightly weaker in neutral (5/8), while Pan-Cytokeratin works best in neutral and acidic environments (8/8), but is less effective in alkaline environments (7/8). Overall, acidic water provided the best retrieval results, particularly for PR and Pan-Cytokeratin, but neutral and alkaline conditions were less efficient. Representative photomicrographs of the immunohistochemical staining obtained with water are presented in Figure 8.

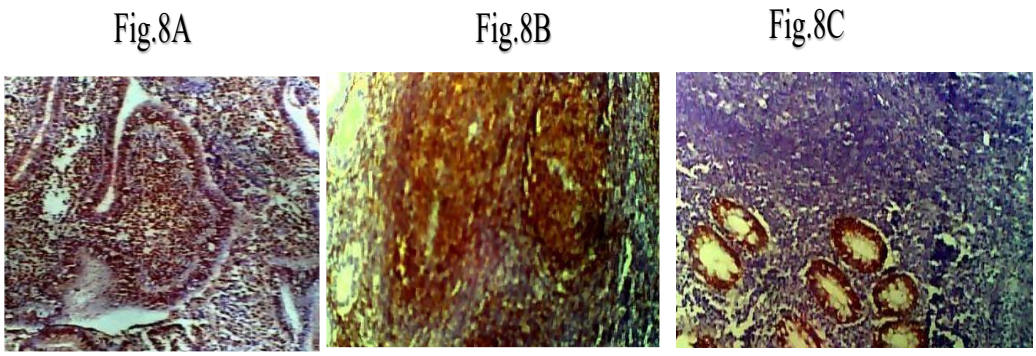


Figure 8. Immunohistochemical staining using water as the antigen retrieval solution. (A) Endometrial tissue stained with progesterone receptor (PR) marker using neutral water. (B) Appendix follicle tissue stained with CD20 marker using neutral water. (C) Appendix tissue stained with Pan-cytokeratin marker using acidic water. All images captured at 10x objective magnification

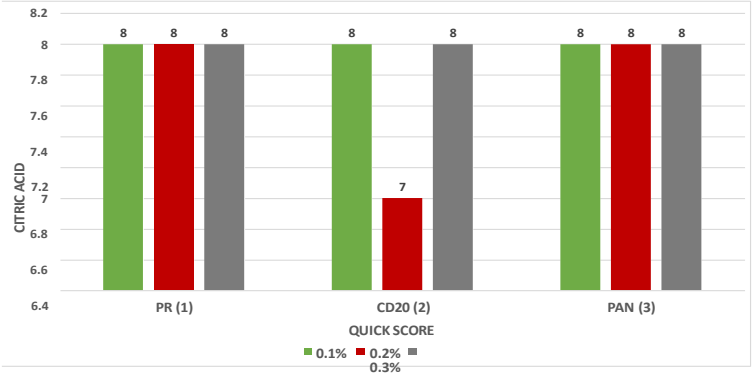


Figure 9. Citric Acid Concentration

Figure 9 Citric acid which was illustrated using the bar chart above and photomicrographs below, citric acid is a highly efficient antigen retrieval solution, giving consistently high quick scores across all three indicators examined. The ideal concentrations appear to be 0.1% and 0.3%, which produced perfect scores (8/8) across all markers. The little decrease in the CD_{20} expression at 0.2% (7/8) implies that minor concentration-dependent fluctuations could influence some antigens, although the difference is not significant. Overall, citric acid has abroad antigen retrieval capacity, making it efficient and a consistent AR solution for immunohistochemistry. Representative photomicrographs of the immunohistochemical staining obtained with citric acid are presented in Figure 10.

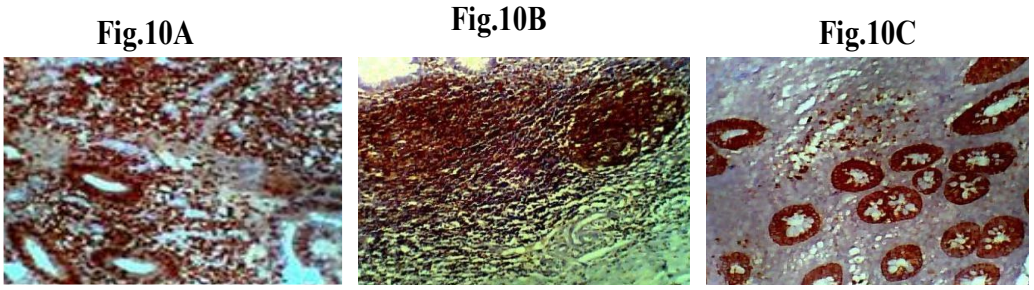


Figure 10. Immunohistochemical staining using citric acid as the antigen retrieval solution across three concentrations. (A) Endometrial tissue stained with progesterone receptor (PR) marker using 0.1% citric acid. (B) Appendix follicle tissue stained with CD20 marker using 0.2% citric acid. (C) Appendix tissue stained with Pan-cytokeratin marker using 0.3% citric acid. All images captured at 10x objective magnification

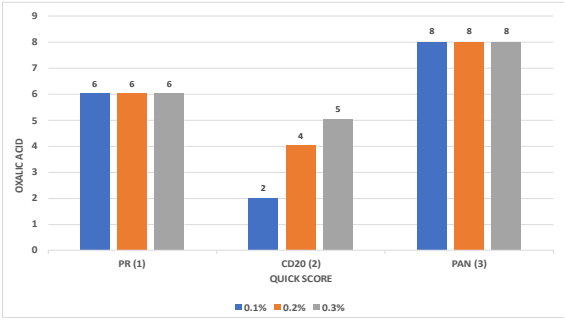


Figure 11. Showed Oxalic Acid Concentration

Figure 11 showed oxalic acid is particularly successful for Pan-Cytokeratin but less effective for progesterone (PR) and CD_{20}, which consistently scored lower than other AR treatments. Oxalic acid's PR remained at 6/8, showing low retrieval abilities. Meanwhile, CD20 retrieval improved with increasing concentrations, but remained very poor (5/8 at best). This shows that oxalic acid may have antigen-specific retrieval properties, as it is powerful for cytokeratin but poor for lymphoid and nuclear markers. Representative photomicrographs of the immunohistochemical staining obtained with oxalic acid are presented in Figure 12.

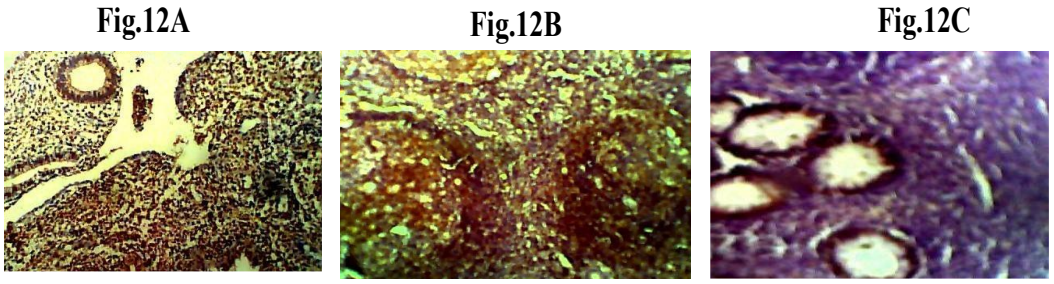


Figure 12. Immunohistochemical staining using oxalic acid as the antigen retrieval solution across three concentrations. (A) Endometrial tissue stained with progesterone receptor (PR) marker using 0.1% oxalic acid. (B) Appendix follicle tissue stained with CD20 marker using 0.2% oxalic acid. (C) Appendix tissue stained with Pan-cytokeratin marker using 0.3% oxalic acid. All images captured at 10x objective magnification

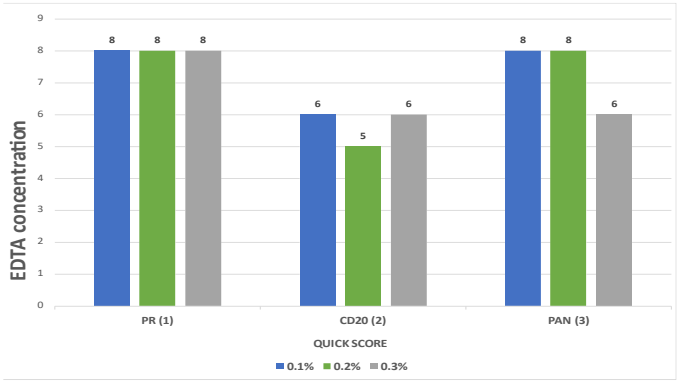


Figure 13. Showed EDTA Concentration

Figure 13 showed the Quick Scores of progesterone (PR), CD20, and pan-Cytokeratin at antigen retrieval concentrations of 0.1%, 0.2%, and 0.3%, as illustrated in the bar chart above and photomicrographs below. Progesterone's (PR) expression was constant (8/8) across all concentrations, indicating persistent antigenicity. CD_{20} staining was highest at 0.1% and 0.3%, but lower at 0.2%, while pan-Cytokeratin staining was greatest at 0.1% and 0.2%, but decreased at 0.3%, indicating possible tissue over-retrieval at higher doses. Overall, the 0.1% antigen retrieval concentration provided the most effective and consistent antigen exposure across all concentration. Representative photomicrographs of the immunohistochemical staining obtained with EDTA are presented in Figure 14.

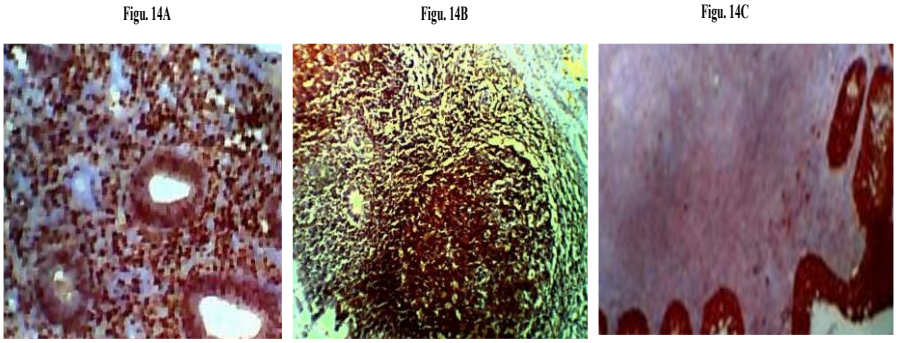


Figure 14. Immunohistochemical staining using ethylene diamine tetra acetic acid (EDTA) as the antigen retrieval solution across three concentrations. (A) Endometrial tissue stained with progesterone receptor (PR) marker using 0.1% EDTA. (B) Appendix follicle tissue stained with CD20 marker using 0.2% EDTA. (C) Appendix tissue stained with Pan-cytokeratin marker using 0.3% EDTA. All images captured at 10x objective magnification

Table 1. pH ranges of Antigen Retrieval Solutions

Antigen Retrieval Solution	0.1% (pH)	0.2% (pH)	0.3% (pH)
Hydrochloric acid (HCL)	3.0	2.5	2.0
Glacial acetic acid	4.5	4.0	3.5
Formic acid	3.5	4.0	3.0
Water	5.0	7.0	9.0
Citric acid	5.5	5.0	5.0
Oxalic acid	2.0	2.0	1.5
EDTA (Ethylene diamine tetra acetic acid)	4.5	4.5	4.0

Table 1; Hydrochloric acid and formic acid showed high quick scores across most criteria, suggesting that a low pH enhances antigen unmasking. EDTA displayed consistent retrieval performance over a mildly acidic range, especially for nuclear antigens while glacial acetic acid, with moderate and rapid scoring, was less aggressive than HCl but not as effective. Formic acid and acetic acid also showed slight differences but remained in the acidic range citric acid maintained relatively steady acidic pH values across concentrations.

The analysis also revealed Oxalic acid showed poor retrieval for some markers, suggesting potential damage to epitopes and that water's pH varied significantly with concentration, ranging from acidic (pH 5.0) to neutral (pH 7.0) to alkaline (pH 9.0). Water's pH instability makes it unsuitable as a standalone solution due to poor retrieval performance and low quick scores compared to buffered solutions.

4.2. Discussion

This study demonstrated that antigen retrieval solutions are vital component in immunohistochemistry staining quality. A comparison of seven AR solutions (hydrochloric acid, glacial acetic acid, formic acid, water, citric acid, oxalic acid, and EDTA), showed significant differences in their ability to improving antigen detection across progesterone receptor (PR), CD_{20}, and Pan-cytokeratin. The research results show variation in staining response when different antigen retrieval solutions were used. This variation is seen from the differences in Quick Score values in the examination of PR, CD20, and Pan-cytokeratin. The results showed significant differences in retrieval performance among the various solutions and concentrations.

The pH table showed that previous studies had found that the pH of antigen retrieval solutions had significant effects on the results and overall effectiveness of immunohistochemical staining. These staining processes need stable acidic or alkaline conditions (Shi et al., 2011). Moderate and stable solutions, such as citric acid, hydrochloric acid, formic acid, and EDTA, provide optimal pH ranges that improve antigen exposure while preserving tissue morphology. Among the acid-based solutions, both hydrochloric acid and glacial acetic acid demonstrated strong performance in retrieval, consistently achieving high scores, particularly for PR and CD_{20}. This suggests that when standard buffers are not available, well-optimized acid-based solutions can serve as effective alternatives. High pH solutions, such as citric acid and EDTA, consistently produced the highest Quick Scores across all three markers (PR, CD_{20}, and Pan-cytokeratin), supporting their status as gold-standard IHC buffers while formic acid showed excellent staining results, with 8 out of 8 samples staining positively for most markers. The retrieval of CD_{20} decreased to 7 out of 8 at a 0.3% concentration, indicating concentration related effects. This supports earlier research that formic acid enhances membrane antigen staining but may not always be more effective than other buffer solutions (Shi et al., 2011).

The differences in staining results cannot be explained solely on the basis of the solution's pH value. Although pH is one of the chemical characteristics related to the antigen retrieval process, the staining response can also be influenced by the type of solution and its chemical characteristics. Therefore, the relationship between pH, type of solution, concentration, and antigen retrieval response needs to be understood carefully based on the pattern of results obtained for each marker.

Water retrieval produced the weakest overall results. Neutral water performed poorly for PR (5/8) and CD_{20} (5/8), but alkaline and acidic treatments substantially improved performance. This suggests that water lacks the buffering ability required to efficiently break down formalin cross-links; however, adjusting the pH can improve its limited retrieval potential (Gamde et al., 2024).

In contrast oxalic acid maintained strong expression for Pan (8/8) but performed poorly for PR (6/8) and CD_{20} (as low as 2/8). This implies it is antigen-dependent and prefers pan-cytokeratin to membrane and nuclear proteins. As a result, oxalic acid is ineffective as a general retrieval solution, with both oxalic acid and water yielding the poorest retrieval outcomes.

However, hydrochloric acid and glacial acetic acid all demonstrated good retrieval efficiency making them suitable alternatives. Formic acid showed satisfactory outcomes that were slightly dependent on concentration. In contrast, oxalic acid and water proved to be the least effective, resulting in weak and inconsistent staining, making them unsuitable for routine diagnostic use. These findings indicate that buffer selection has significant effect on IHC outcomes, and that optimum antigen retrieval should be adjusted to the appropriate pH, concentration, antigen of interest and type of tissue.

Overall, the data clearly demonstrate that citrate and EDTA buffers are superior for antigen retrieval in immunohistochemistry (IHC), with hydrochloric acid buffer closely following as an effective solution. Additionally, the Quick Score technique is a practical and reproducible method for evaluating the performance of antigen retrieval solutions. This study also contributes to further efforts to standardize IHC methods, which are essential for enhancing diagnostic accuracy and reproducibility across laboratories.

5. Conclusion

Based on the findings of this study, antigen retrieval is crucial in immunohistochemistry, especially for formalin-fixed, paraffin-embedded tissue. The appropriate retrieval solution and factors dramatically improve antigen exposure and antibody binding. Citric acid and EDTA demonstrated the most consistent and efficient antigen recovery across PR, CD20, and Pan-Cytokeratin thus reinforcing their status as immunohistochemistry gold-standard buffers. Further study would exclude variables in immunohistochemistry protocols such as antibody selection and detection methods. Based on the research results, the use of different antigen retrieval solutions showed variation in immunohistochemical staining response for PR, CD20, and Pan-cytokeratin. Citric acid showed a relatively consistent staining pattern under the conditions of this research. This finding indicates that the selection of the type of solution and the antigen retrieval concentration is an important aspect in obtaining appropriate immunohistochemical staining results. Further research is needed to evaluate the consistency of the results using a wider variation of tissues, markers, and procedural conditions.

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