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A COMPARISON OF THE EFFECT OF COMMERCIAL pH BUFFERS ON EPITOPE RETRIEVAL OF 'HER-2/*neu*' PROTEIN

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ABSTRACT

Aim: The significance of immunohistochemistry standardization became important with the disclosure of alarmingly high records of inconsistent results in the assessments of HER-2/neu expression status in women with breast cancers. Formalin-Fixed-Paraffin-Embedded tissue usually presents with conformational macromolecular changes, masking the epitope with sub-optimal staining of HER-2/neu protein. The pH of epitope retrieval solution appears to significantly influence the completion of the unmasking process. The present study therefore examines the impact of commercial buffers on epitope retrieval of 'HER-2/neu' protein so as to optimize diagnosis and management of breast cancers.

Methods: The study examined three pH buffers, non-retrieved sections and distilled water as a control on a 3+ HER-2/neu FFPE tissue block by water-bath epitope retrieval procedure and subsequently stained immunohistochemically.

Results: The pH of the buffers does not significantly influence the staining quality of HER-2/neu protein. However, pH 6 appears to be the most optimal as associated with other buffers examined.

Conclusion: pH 6 buffer in conjunction with water-bath procedure is strongly recommended as the first-choice buffer for epitope retrieval of HER-2/neu protein.

Keywords: pH, Buffers, Epitope Retrieval, HER-2/*neu*, Immunohistochemistry

INTRODUCTION

The dire significance of Immunohistochemistry (IHC) standardization became apparent with the disclosure of alarmingly high records of discordant outcomes in assessments of Human Epidermal Growth Factor Receptor-2 (HER-2/neu) expression which is key in taking treatment decisions for women with breast cancers. HER2-positive status represents a major therapeutic target, since HER-2/neu overexpression acts a central role in oncogenic alteration and tumourigenesis (Chazin, 1992), and is related to poor prognosis with shortened disease-free and general survival (Slamon, 1987). HER2/neu-positivity alone confers intermediate or high-risk status (Goldhirsch *et al*., 2006). Women whose breast cancers overexpress HER2/neu protein, i.e., 20-30% of breast cancers (Penault-Llorca *et al*., 2005), with immunohistochemistry score of 3+ gain the most clinical benefit from Herceptin (trastuzumab). It is vital that assessment of HER-2/neu status must be accurate and of high quality. It is indeed necessary to accurately identify those patients who will profit from this management while avoiding futile treatment of patients who are unlikely to respond. False positive HER-2/neu results can give rise to over-treatment of patients, raise false hope and waste resources while false negative results may deny women with breast cancer potentially life-extending treatment. Central to immunohistochemistry of HER-2/*neu* is the reaction of its antibody (monoclonal or polyclonal) with the matching epitope(s) on its antigen. This reaction is usually threatened due to precipitation reaction ensuing from formalin fixation and paraffin processing on the HER-2/*neu* protein yielding cross-links (Dapson,

2007), hence, masking the HER-2/*neu* epitopes. For an optimal HER-2/*neu* antigen/antibody complex to be established, the epitopes have to be unmasked. The procedure for epitope retrieval (ER) centered on heating to elevated temperatures is acknowledged to be useful for an efficient immunostaining of formal-fixed paraffin-embedded (FFPE) tissue sections (Igarashi *et al*., 1994). SDS-PAGE studies post heating the formaldehyde-treated protein, indicated that the core mechanism of heat-induced epitope retrieval (HIER) is centered on interfering with methylene bridges created by formaldehyde fixation (Emoto *et al.,* 2005). Heating, besides reversing epitope masking produced by formaldehyde in formalin-fixed paraffin sections, also uncover epitopes concealed due to molecular configuration of native molecules in frozen sections (Kakimoto *et al.,* 2008). Specifics of the mechanisms responsible for HIER is still vague but (Yashimata and Okada, 2005) suggested that the pH of retrieval buffer is critical for appropriate refolding of antigens so that they are capable of reacting with antibodies. Cancer testing facilities in Nigeria do not have a standardized pH buffer for HER-2/neu epitope retrieval. Commonly used buffers are any of these three (pH 6, 8 and 9). Therefore, the influence of pH on epitope retrieval of HER-2/neu protein employing water-bath technique may not have been adequately explored. This study is thus significant to evaluate the optimal pH for epitope retrieval of HER-2/neu protein in breast cancers. In this study, three buffers of different pH were examined to determine which gave the most optimal demonstration of HER-2/neu protein.

Working Hypothesis

- **H_0**: The occurrence and re-occurrence of the high level of discordance in HER-2/neu testing results are NOT associated with pH variation of its epitope retrieval buffer.
- **H_1**: The emergence and re-occurrence of the high degree of discordance in HER-2/neu testing results are related to the pH change of its epitope retrieval buffer.

MATERIALS AND METHOD

Tissue Block

Formalin Fixed (10% Neutral Buffered Formalin) Paraffin-Embedded (FFPE) tissue block was retrieved from the archive of the Department of Pathology, Ahmadu Bello University Teaching Hospital, Zaria, Nigeria.

Antibody Biomarker and Reagents

A rabbit monoclonal antibody (Clone GR011) against HER-2/*neu* used at a dilution of 1 in 80, (Genemed Biotechnologies South San Francisco, USA). Retrieval buffers pH 6, pH 8 and pH 9 (Leica, Germany).

Section Preparation

The tissue block with a 3+ IHC score was sectioned at 3µm, mounted on Silane coated slides (Surgipath X-tra Slides sourced from Leica Biosystem). The slides were labeled as 5 groups of pH 6, 8, 9, distilled water as a control and a non-retrieved group (NR). Slides were baked at 100°C for 3 hours and taken to distilled water.

Preparation of Buffers

10X (stock solution) were diluted to 1X (working solution) using fresh distilled water into pre-labeled retrieval plastic jars. The pH of the respective buffers was validated using the pH meter (Hanna). A fourth jar containing distilled water was labeled as well.

Procedure for Epitope Retrieval

The plastic jars containing the epitope retrieval buffers were heated in the water-bath (DK-420, China) to 65°C. The slides were totally immersed in its corresponding pre-labeled jars and heated to 95°C, and the heating was maintained for 20 minutes at 95°C. The jars containing the section slides were cooled to room temperature for a further 20 minutes.

Immunohistochemical Staining Procedure

Sections labeled pH 6, pH 8, pH 9, distilled water and NR were covered in peroxidase block (Genemed, U.S.A.) to destroy endogenous peroxidase activity for 10 minutes and incubated with HER-2/neu antibody (Genemed, U.S.A.) diluted 1:80 for 60 minutes, rinsed in PBS wash (Genemed, U.S.A.), the sections were incubated with mouse plus rabbit linker and with horseradish peroxidase (Genemed, U.S.A.) for 15 minutes each followed by 2 washes of 2 minutes each. The development of the chromogen was 5 minutes with 3,3'-diaminobenzidine hydrochloride (DAB) mixed equally with its substrate solution (Genemed, U.S.A.). Slides were then counterstained with Haematoxylin solution (Jallica Scientific, Zaria), drained and mounted with DPX.

Sections Evaluation for Immunoreactivity

Staining immunoreactivity was categorized as 0, no immunoreactivity; 1+, incomplete membrane staining within < 10% of the invasive tumour cells; 2+, for incomplete circumferential membranous staining in > 10% of the invasive tumour cells and 3+, for complete intense circumferential membrane staining in > 30% of the tumour cells (Wolff *et al*., 2013). Individual sections were assessed and evaluated by two trained medical laboratory scientists (JOE, JID), inconsistencies were deliberated until harmony was established. Appraisals of immunostaining scores were carried out at a matching locus on each stained section so as to circumvent any discrepancy due to the heterogeneity of protein distribution.

RESULTS

The outcomes are as abridged in Tables 1, 2 and 3.

Table 1**:** Dataset on influence of pH Buffers in epitope retrieval of 'Her-2/Neu' protein

REPLICATIONS	pH 6	pH 8	pH 9	Distilled water	NR SECTIONS
1	3+	2+	3+	2+	1+
2	3+	2+	2+	2+	1+
3	3+	2+	2+	3+	1+
4	3+	3+	3+	3+	3+
5	3+	2+	2+	1+	2+
6	2+	2+	2+	2+	2+
7	2+	3+	2+	2+	2+
8	3+	2+	3+	2+	2+
9	2+	3+	3+	3+	3+
10	2+	2+	3+	2+	2+
11	3+	2+	2+	1+	2+
12	3+	3+	3+	2+	2+

Table 2: Mean and Standard Deviation of the different pH Buffers, Distilled Water as control and Non-Retrieved Sections

pH 6 (Mean SD)	pH 8 (Mean SD)	pH 9 (Mean SD)	Distilled Water (Mean SD)	NoN-Retrieved Sections (Mean SD)
2.666± 0.492	2.330± 0.492	2.500± 0.522	2.080± 0.669	1.917± 0.669

Table 3: Hypothesis Test Summary (Chi-square/one-sample Binomial) tests using SPSS21

	Null Hypothesis	**Test**	**Significance**	**Decision**
1	The categories defined by pH6 = 3 and 2 occur with probabilities 0.5 and 0.5	One-Sample Binomial Test	0.388[1]	Retain the Null hypothesis
2	The categories defined by pH8 = 2 and 3 occur with probabilities 0.5 and 0.5	One-Sample Binomial Test	0.388[1]	Retain the Null hypothesis
3	The categories defined by pH9 = 3 and 2 occur with probabilities 0.5 and 0.5	One-Sample Binomial Test	1.000[1]	Retain the Null hypothesis
4	The categories of distilled water occur with equal probabilities.	One-Sample Chi-Square Test	0.174	Retain the Null hypothesis
5	The categories of Non-Retrieved Sections occur with equal probabilities.	One-Sample Chi-Square Test	0.174	Retain the Null hypothesis

Asymptotic significances are displayed. The level of significance is 0.05. [1]Exact significance is displayed for this test.

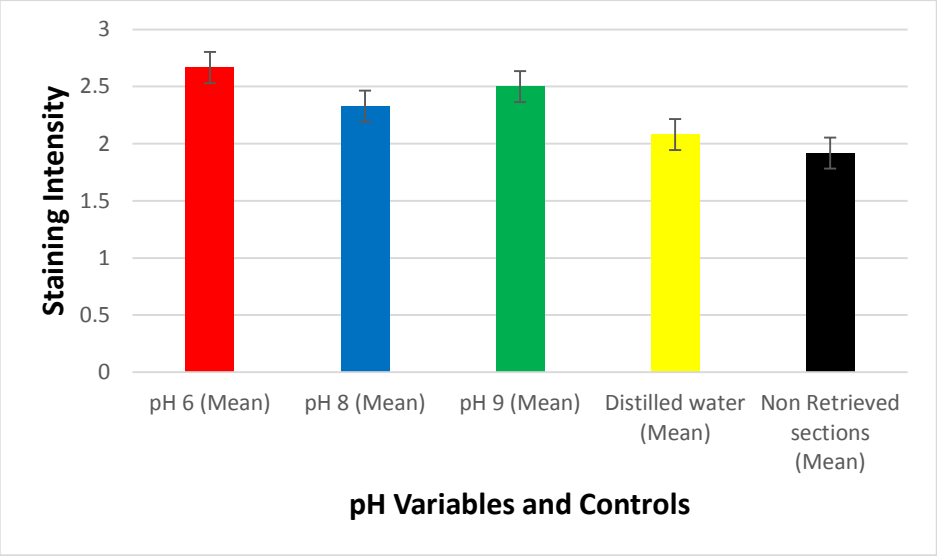

Figure 1: Simple Bar Chart showing the relationship between mean staining intensity of HER-2/neu protein and different pH Buffers, Distilled Water as control and Non-Retrieved Sections.

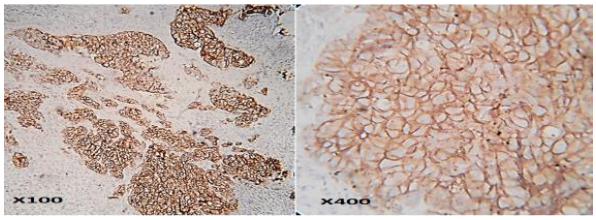

Plate 1: HER-2/neu stained sections retrieved at pH 6 (X100 and X400)

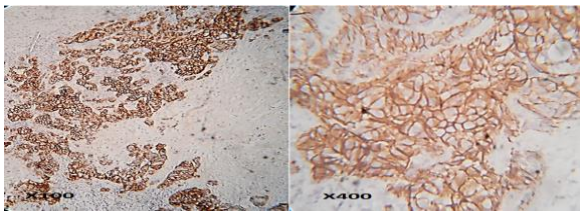

Plate 2: HER-2/neu stained sections retrieved at pH 8 (X100 and X400)

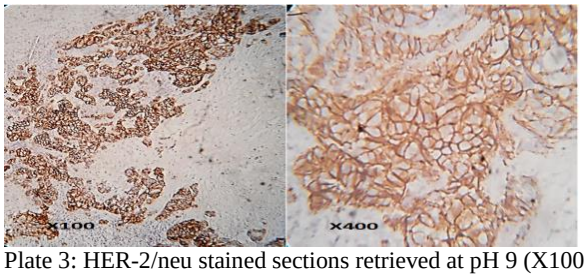

Plate 3: HER-2/neu stained sections retrieved at pH 9 (X100 and X400)

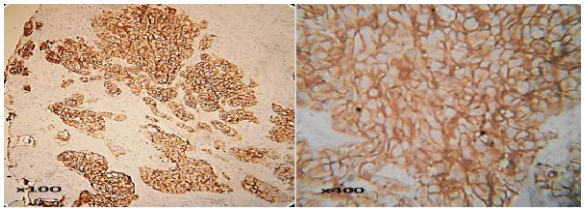

Plate 4: HER-2/neu stained sections retrieved using distilled water (X100 and X400)

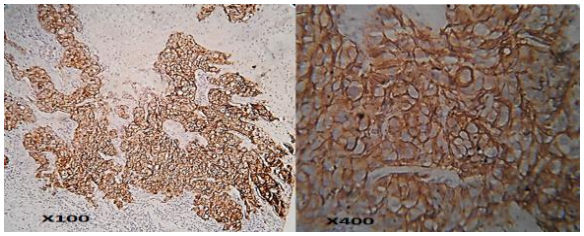

Plate 5: HER-2/neu non-retrieved stained sections (X100 and X400)

Immunohistochemical stained sections of the FFPE HER-2/neu 3+ tissue block show an insignificant difference in the circumferential staining intensity with all the retrieval buffers employed except for pH 6 (Plate 1) which displayed a little more circumferential staining intensity.

DISCUSSION

Buffer at pH 6 has been reported to be the most popular solution for HIER (Morgan *et al.*, 1997). This is inconsistent with (Emoto *et al.*, 2005), which reported that Citrate buffer is not an ideal retrieval solution for a majority of antigens and (Gray *et al.*, 2007) reported the best quality staining with Dako buffer pH 9. This study established the highest mean staining value (2.666) at pH 6 probably due to less

entangling of the tissue protein at acidic pH ionic strength; followed closely by pH 9 (2.500) also probably due to more entangling of tissue protein presumably caused by increased hydrophobic forces; pH 8 (2.330) and non-retrieved sections produced the least mean staining value (1.917). Distilled water at pH 7 yielded a low mean staining value of (2.080) due to a highly entangled tissue protein, triggered by the concurrent existence of ionic and hydrophobic forces. A little mean staining value of 1.917 produced by Non-Retrieved Sections as against 2.666 and 2.500 produced by pH 6 and pH 9 respectively emphasizes the need for epitope retrieval for HER-2/neu staining, consistent with (Kim *et al*., 2004) where distilled water was used in place of a retrieval buffer solution by both microwave and pressure cooking methods, which resulted in a negative HER-2/neu status. Since it was established that demonstration of HER-2 amplification and over-expression in breast tumours is required to define eligibility for Trastuzumab therapy, non-optimization of the retrieval pH buffer employed coupled with other factors could lead to a false negative or equivocal HER-2 status. This makes the patient ineligible for Herceptin therapy, eventually denying women with breast cancer potentially life-extending treatment. Test for the Null Hypothesis to establish the no-significant difference on the influence of pH buffers on epitope retrieval of HER-2/neu protein suggest the retention of the null hypothesis as seen in Table 3. This implies that the differences in HER-2/neu staining intensity are not significant.

Conclusion

The pH of the retrieval buffers studied does not significantly influence the staining quality of HER-2/neu protein. However, pH 6 buffer appears to be optimal for epitope retrieval of HER-2/neu protein.

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