Comparison of Diagnostic Fine-Needle Aspiration of Breast Masses Obtained from Cell Blocks and Conventional Smear Methods Using Prognostic/Predictive Biomarkers for Confirmation.

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ABSTRACT

Background: Fine-needle aspiration cytology (FNAC), a rapid, minimally invasive technique has been extensively used as the first-line diagnosis of breast lesions. However, sometimes its yield is not adequate for precise diagnosis and the risk of false-negative and indeterminate diagnosis is always present. **Objectives:** The aim of this study is to compare the diagnostic results of fine needle aspiration cytology (FNAC) of breast mass samples obtained from cell blocks (CB) and smears (CS), using ER, PR, and HER2/neu as objective tumour makers for confirmation. **Method**: Smears were made from breast aspirates collected from 150 consenting female participants at the FNAC clinic of the Department of Anatomic and Molecular Pathology, Lagos University Teaching Hospital (LUTH), using the conventional smear method. A modified cell block (CB) preparation was also prepared using 10% Neutral Buffered Formalin (NBF) Both smears and sections from the cell block preparations were stained in haematoxylin and 1% aqueous eosin stain and demonstrated for estrogen (ER), progesterone (PR) and human epidermal receptor 2 (HER2) tumour markers using standard immunocytochemical (ICC) methods. A comparison was then made of the final diagnosis of both methods. **Results**: The CS method showed 6(4%) of the participants were C_5 malignant, 3(2%) were C_4 suspicious for malignancy, 114(76%) C_2 benign lesion, 6(4%) had blood/mucus exudates 15(10%) inadequate smears and were C_1 unsatisfactory, while the remaining 6(4%) had acellular smears. However, the cell block (CB) method showed that 15(10%) of the participants had inadequate cells C_1 unsatisfactory, 6(4%) were inflammatory C_1, 114(74) were C_2 benign, 9(6%) were suspicious/benign, while the remaining 9(6%) were malignant as against 6(4%) of the conventional smear. The IHC method carried out on the additional 3(2%) yield from the cell block method using ER, PR, and HER2/neu antibodies revealed that 1participant was ER, PR, and HER2/neu Positive, while the other 2 were negative for the 3 antibodies used. **Conclusion:** Cell block method with biomarker gave better architectural patterns, high cellularity, morphological features and an additional yield of malignant cells than the smear method.

INTRODUCTION

Although open surgical biopsy is the 'gold standard' for diagnosis and management of palpable breast lesions, in recent years fine-needle aspiration (FNA) a minimally invasive technique commonly used to obtain pathologic material to arrive at diagnosis in solid organ malignancies has attracted much attention as a simple, safe, rapid, cost-effective, and accurate diagnostic method (1,2,3). FNAC had been widely used in the diagnosis of breast tumors, thyroid tumors and other palpable lumps which have been detected, if the lump cannot be felt, imaging may be required to find the exact location (3, 4).

It is an important diagnostic tool in the evaluation of triage of patients with lymphadenopathy. It also offers a simple and inexpensive test for diagnosis of reactive hyperplasia, infections (which could be caused by fungi, virus, bacteria, Chlamydia and mycobacterium), granulomatous lymphadenopathies and metastatic diseases (5, 6, 7). The architectural arrangement of the cells in the smear provides robust information about the histology of the tissue from which the sample was removed (5).

Fine needle aspiration of breast mass using the smear technique has been shown to be a safe and accurate technique, it allows the visualisation of the cells aspirated from the lesion, in many circumstance it shows high accuracy, sensitivity, and specificity (8) fine needle aspiration of cytology smear is a test and should be interpreted with the entire clinical, radiological, and pathological information in every circumstances to avoid error, false-negative and false-positive results have been reported in some series because there are instances where the differentiation of benign and malignant is not possible, and this may be due to paucity of specimen sampling, inflammatory background or there is morphological overlap of both lesions (e.g., atypical hyperplasia and low-grade carcinoma in situ) (9).

In some instances, aspirated cells can be evaluated by flow cytometry, cell block preparation or with immunologic markers used on the smear or cell block (9. 10). American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines recommend that immunologic markers such ER, PR, HER2 etc, because of their increasing clinical importance be performed on the lesion as to determine the hormone receptor status of the breast lesion after validation of the biomarker (10,11, 12). But the use of cytologic samples for determining a patient's estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) status are yet to be validated, in part due to the decline in breast fine needle aspiration cytotology smear preparation (13). Cell blocks prepared from residual tissue fluid have been shown to assist in further establishing a more definitive cytopathologic diagnosis, making it a useful technique complimentary to FNAC. In this study we compared the diagnostic results of FNA obtained from cell blocks which permits sectioning for routine pathologic staining, molecular testing and IHC which is valuable in identifying distinctive immunoreactive profiles of various cell types, adding greatly to diagnostic accuracy with cytologic smears which include bloody yields that often obscure cell morphology and the relative difficulty of applying adjunctive immunocytochemical testing because of loss of cells and molecular studies when the aspirated material is entirely submitted

as smears (14), in the evaluation of female breast masses using ER, PR, and HER2/neu as objective tumour makers for confirmation of positive cases.

MATERIALS AND METHODS

A total of 150 fine needle aspiration samples were collected for over a period of 12 months from female participants between the age range of 16 – 75 years with palpable breast masses at the FNAC clinic of the Department of Anatomic and Molecular Pathology Lagos University teaching Hospital, after Informed consent has been obtained from the women and ethical clearance obtained from the Lagos University Teaching Hospital Health Research and Ethics Committee (Luthhrec) dcst/luthhrec/vol.xvi/2652

Specimen collection

The samples were collected using a 10-mL (meno-ject) disposable hypodermic syringe with an attached 23-gauge needle (u-mec, k0941) inserted just under the skin surface and negative suction was applied to the syringe. Multiple passes were made into the mass without exiting the skin surface. Six passes through the mass were made, the negative pressure created was maintained on the syringe in order to obtain enough pathologic material for processing, the aspirated sample in the needle was pushed out on to two clean glass slides for the preparation of cytology smears. The smears were immediately placed in a coplin jar filled with 95% ethanol and was left to fix, after which they were stained using Haematoxylin and Eosin (H&E) stain (15). The remaining sample in each of the needle and syringe was expelled into a sterile universal bottle containing 10 mls of 10% neutral buffered formalin (NBF) according to the ASCO/CAP guidelines (16). The 10% neutral buffered formalin was drawn up through the needle into the syringe and then gently ejected back into the universal container and submitted for cell-block analysis.

Cell block preparation

The principle is based on the fixation, histological processing of sediments of aspirates or fluids collected from patients, and the subsequent paraffin embedding of the cells (16).

The cell block (CB) was prepared by firstly, concentrating the cells together by mixing, using a vortex XH-B to resuspend the cells, and then spinning the specimen in a centrifuge (Liqui-prep centrifuge LP500) for 10minutes at 1400rpm.The supernatant was decanted, and the cell button (Residue) was carefully removed, placed on a lens paper, tinged with few drops of eosin to colour it and render it more visible, wrapped in the lens paper, and placed in a pre-labelled tissue cassette. It was then processed histologically, using automatic tissue processor Leica TP 1020 (Leica Microsystems, International, Nussloch, Germany) 13- hour processing schedule as follows: 80% ethanol with 1 change (2 hours); 90% ethanol (2 hour), 95% ethanol, 2 changes (1 hour each), 3 isopropyl alcohol (1 hour each); xylene, 2 changes (1 hour each), paraffin wax, 60°C (1.30 hour); and paraffin wax, 60°C for impregnation for 1hour. The cell blocks were embedded in paraffin and sectioned at 3 μm thicknesses using rotary microtome (RM2255, Leica Microsystems) to produce two slides from each block and stained with haematoxylin and eosin (H&E). The slides were deparaffinised, hydrated and stained with Cole's haematoxylin for 5 minutes, it was then rinsed in water, differentiated in 1% acid-alcohol for 3 seconds, rinsed in water, blued in Scott's solution for 2 minutes and counterstained in 1% eosin for 1 minute, they were then dehydrated through ascending grades of alcohol, cleared in xylene and cover slipped using DPX (17). Both the smear and the cell block of each of the 150 case were reviewed and the diagnosis confirmed using the (NHSBSP) 5 tier guidelines.
In all the cases reviewed 3 participants were screened as positive for malignancy. Immunocytochemistry (IHC) stain was carried

out on the 3 samples of the participants that were positive using ER, PR, HER2 antibodies.

Immunohistochemical staining (IHC)

For IHC staining, the monoclonal antibody ER, PR, HER2 antibodies clone (Dako, Glostrup, Denmark) were used, Labvision detection kit (Labvision, Fremont, CA, USA) was also used for the staining following the manufacturer's instructions.

Antigen Retrieval

The sections were placed on a superfrost glass slides, incubated overnight at 37°C, dewaxed, hydrated, a microwave boiling AR method was used for Pre-treatment with citrate buffer pH 6.

Immunohistochemical Analysis

Before the staining, the sections after retrieval were set at room temperature for 20 minutes and washed with PBS. Methanol–hydrogen peroxide was used to block endogenous enzyme for 10 minutes. Normal goat serum was used to block non-specific binding reactions as appropriate for 10 minutes.

The blocking solution was drained off from the slides and the appropriately diluted primary antibody was applied on to the sections on the slides and incubated for 1hr at room temperature. The secondary antibody (biotinylated anti-mouse immunoglobulin) and label (avidin-biotin complex) were incubated for 30 and 45 minutes at room temperature, respectively. A wash step with PBS at a neutral pH for 2 changes at 5 minutes was carried out between each step of these immunohistochemical staining. The chromogen used to reveal the color of the antibody staining was diaminobenzidine tetra hydrochloride. Slides were counterstained for 1 minute with Mayer haematoxylin. Slides with known positive reaction for each tested antibody were used as positive control slides, and slides with AR treatment were used as negative control slides by replacing primary antibodies with PBS to confirm the staining results. To avoid potential variations among different batches of immunohistochemical

staining procedures, all slides tested with each individual antibody were completed in a single "run" for more accurate comparison (17).

After processing and staining the slides, a blind review of the slides of all the 150 female participants that consented in the study was conducted and diagnosis confirmed for both the smear and cell block technique. The slides were studied in detail taking into account the available clinical data, investigation reports such as ultrasound scan and mammographic reports as well as the morphological details. Result obtained was categorized as benign, suspicious for malignancy and malignant lesions. The various morphological criteria that were taken into account included the cellularity, arrangement of the cells (acini, papillae and cell walls) and the cytoplasmic and nuclear details, various variables such as history of breast lump, duration etc. All these criteria were put together and used for classifying the various cytomorphological patterns.

The cases were reported using the 5-tier diagnostic categories in accordance with United Kingdom – National Health Scheme Breast Screening Programme (NHSBSP) guidelines, using this guideline, C1 - is Insufficient cells for cytological analysis, i.e. fewer than five epithelial cell groups, C2 - Cells present all benign; no suspicious features, C3 - Cells suspicious but probably benign, C4 - Cells suspicious but probably malignant and C5 - Definitely malignant Ibukunle (18, 19)

Evaluation of Immunohistochemical Staining Results

Immunostained slides were also evaluated by 2 observers independently in a blinded manner by light microscopy. Tumours were scored as positive for Estrogen receptor (ER) and Progesterone receptor (PR) when nuclear staining was identified in at least 10% of cells and when definite or entire membrane staining was seen in Her2.

Statistical analyses

The data generated were analyzed using Spearman rank-order correlation coefficient a nonparametric measure of the strength and direction of association that exists between two variables measured on at least an ordinal scale, It assesses how well the relationship between two variables can be described using a monotonic function.

RESULTS
A comparative evaluation of the smear versus the cell block technique was conducted. Out of the 150 patients, only 10% had history of breast Lump, 4% indicated that their mother had breast lumps, 3.3% indicated that their niece had breast lumps, and 2.7% indicated that their sister to have had breast lumps in the past. 12 of the participants had lumpectomy about 3 months earlier before coming for the fine needle aspiration. The smear stained with the Haematoxylin and 1% Eosin method revealed clear, sharp and good architectural patterns of the cells, but the cell block technique concentrated the cells more, showed clearly recognizable normal and abnormal cells with little or no shrinkage. Its cytomorphologic features were well maintained, and the staining characteristics of the nucleus, nucleoli, and cytoplasm were sharp and crisp with clear recognition of nuclear and cytoplasmic features. 3 participants were screened as positive for malignancy which indicates their presentation with breast cancer. One of those 3 was diagnosed only from the cell block technique as positive and as C2 benign in the smear technique. The other two were diagnosed in both techniques. Immunocytochemistry test was carried out on the 3 participants that were positive using ER, PR, HER2 antibodies to aid their staging, prognosis and management, and one of them was ER, PR, HER2 positive, while the remaining two were ER, PR, HER2 negative. 34 participants had C2 benign lesion in both smear and cell block technique. 4 were diagnosed as C3 benign; suspicious for malignancy in the cell block technique and as C2 benign with very scanty cells in the conventional smear method. 2 participants presented with inflammatory cells in the cell block technique and acellular in the smear. 2 participants had bloody cells in the smear method and C2 benign in the cell block technique. Inadequate cells were obtained in 5 participants to make a concrete diagnosis in both techniques, while the last participant was diagnosed as having a C4 benign lesion that is highly suspicious for malignancy in the smear technique and as C2 benign in the cell block method

Table1: Shows the descriptive statistics of the age range of the patients to be between 16 and 75 years with a standard deviation of 15.8, which indicates an insignificant dispersion of individual age from the mean age. Participants duration of lump was from zero to about seven years (or 84 months), with the mean duration of 11 months. Also, the sizes of the lumps range from 4cm^2 to 360 cm^2 combined, with a mean of 26.24cm and standard deviation of 35.43. The Smear technique in Table 2 shows that 10% of the outcome were inadequate and were graded as C1, 4% were bloody Cells and were graded as C1 unsatisfactory. Another 4% were acellular and were graded as C1, making the total of about 18% of C1 as shown in the cumulative percent. Majority of the result shows 76% C2 benign, with 2% showing result of C4 (Suspicious for malignancy) and 4% being cancerous in nature (C5 malignant). While in Table 3: The Cell block technique indicated about 74%, representing about 111 patients to be

benign, 10% representing 15 patients were C1 (inadequate), 6% are C3, with another 6 % found to be C5 malignant, while a few 4% were C1 (inflammatory). The analysis from the cumulative frequency shows that about 6% of the results were malignant. The smear diagnosis tends to be less effective when compared with cell block diagnosis, as Cell block diagnosis was able to reveal about 6% of malignancy (C5 Malignant), while smear diagnosis was 4% of malignancy. Table 4: Shows a weak negative correlation (-0.080) between cell block technique and smear technique, this indicates that the cell block technique had more C5s and

C4s, while the smear technique had more C1s and C2s. This result further justifies the outcomes on tables 4.5 and 4.6 that the cell block technique seems to be more effective that smear technique, even though the 1-tailed and 2-tailed hypotheses show that the correlation is not very significant as the p-values (0.166 and 0.332) are greater than the traditional 1 percent (0.01) and 5 percent (0.05) level of significance.

In general, the cell block technique gave a high cellularity, better architectural patterns, morphological features and an additional yield of malignant cells than the smear,.technique.

Table 1: Descriptive Statistics of the participants in relation to mass greater than 2cm

Variables	N	Minimum	Maximum	Mean	Std. Deviation
Age (yrs)	150	16	75	32.95	15.782
Duration of Lump (Months)	150	0	84	11.66	18.337
Size of Lump (cm^2)	150	4.00	360.00	26.2433	35.42815
Previous Lumpectomy (Months)	150	0	48	2.78	10.587
Valid N (list-wise)	150		-	-	-

Table2: Smear diagnosis in percentage

S/N Grading	Frequency	Percentage	Cumulative Percentage
C1(Inadequate)	15	10.0	10.0
C1(Bloody cells)	6	4.0	14.0
C1(Acellular)	6	4.0	18.0
C2 Benign	114	76.0	94.0
C4 Suspicious	3	2.0	96.0
C5 Malignant	6	4.0	100.0
Total	150	100.0	

Table 3: Cell block diagnosis in percentage (%)

Grade	Frequency	Percent	Cumulative Percent
C1 (Inadequate)	15	10.0	10.0
C1(Inflammatory	6	4.0	14.0
C2 benign	111	74.0	88.0
C3 Susp / benign	9	6.0	94.0
C5 Malignant	9	6.0	100.0
Total	150	100.0	-

KEYS : C1 – Unsatisfactory, C2 – Benign, C3 - Suspicious, probably benign
         C4 – Suspicious for malignancy, C5 – Malignant

Table 4: Correlations between Cell block & Smear technique using the spearman's rho

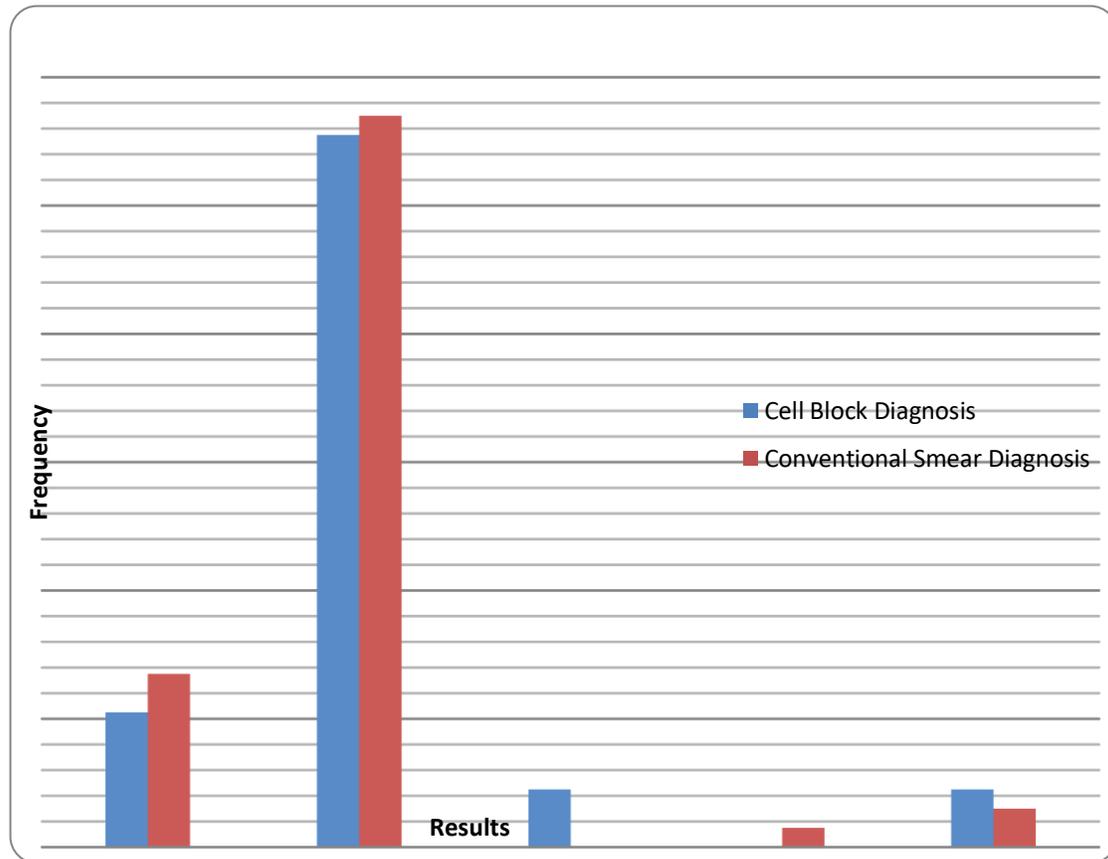|	Factor		Cell block diagnosis	Conventional smear diagnosis
Spearman's rho	Cell block technique	Correlation Coefficient	1.000	-.080
		Sig. (2-tailed) Sig. (1-tailed)	.	0.332 0.166
		N	150	150
	Conventional smear technique	Correlation Coefficient	-.080	1.000
		Sig. (2-tailed) Sig. (1-tailed)	0.332 0.166	.
		N	150	150

Figure 1: Bar chart comparing the two techniques

The above comparison shows that both techniques were diagnostic with their highest percentage at C2 benign. The smear technique had 2% more than cell block technique. 14% of the cell block diagnosis was C1 (unsatisfactory), while those of smear diagnosis were 18%. About 6% were C3 (suspicious, probably benign) for Cell block diagnosis, while Smear diagnosis had nothing. 2% of the conventional smear diagnoses were suspicious for malignancy (C4), while none of cell block diagnosis was suspicious for malignancy (C4). Finally, about 6% of cell block diagnoses were malignant (C5), while only 4% were C5 malignant in the smear diagnosis results. Thus, the cell block technique was able to reveal more malignancies than smear technique
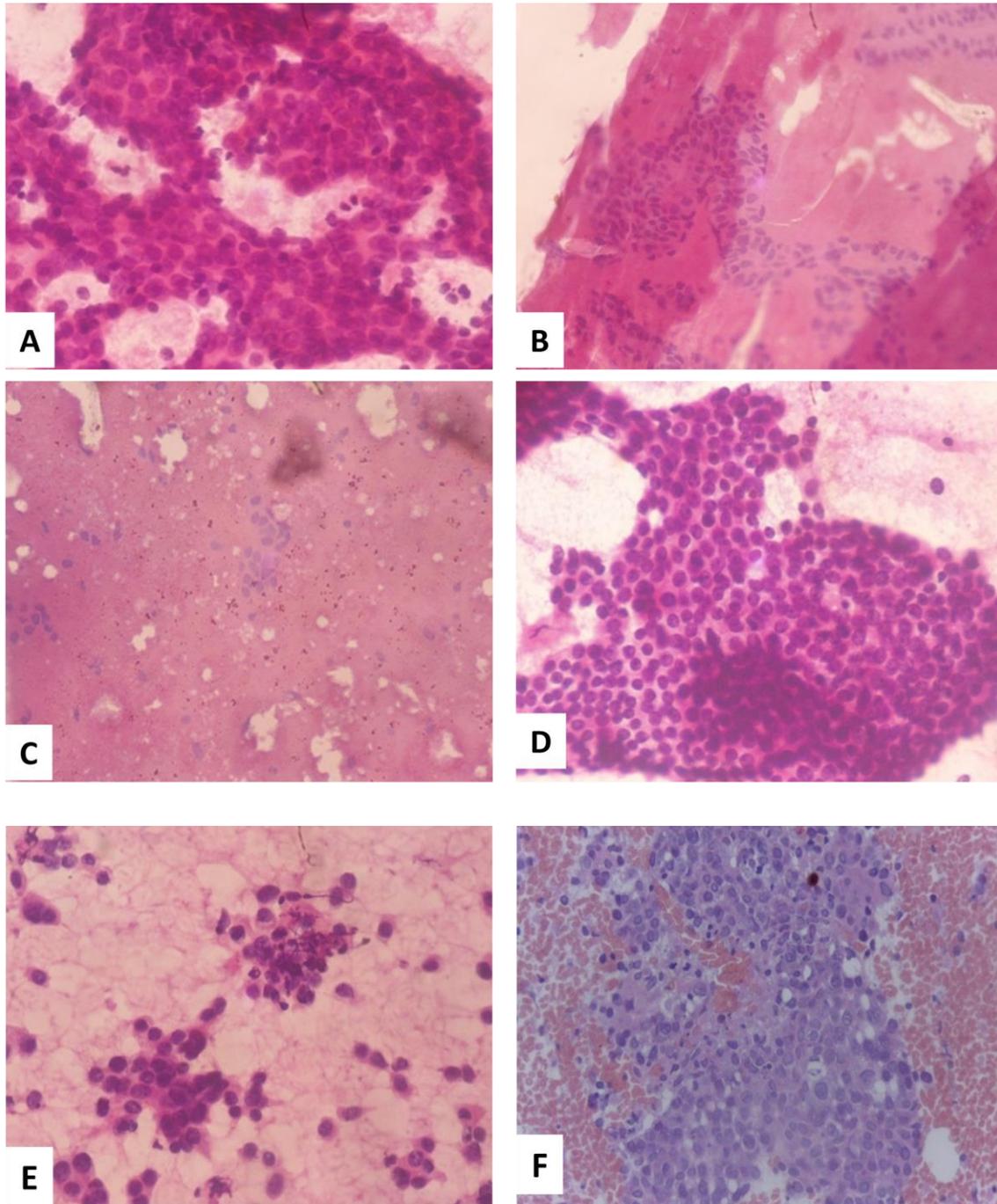
Figure: 2 Photomicrograph showing slide A and B smear preparations diagnosed as C2, slide C and D C4 Benign & highly suspicious for malignancy, while slides E and F show smear and cell block preparation diagnosed as C₅ Malignant. Haematoxylin & Eosin stain. X400
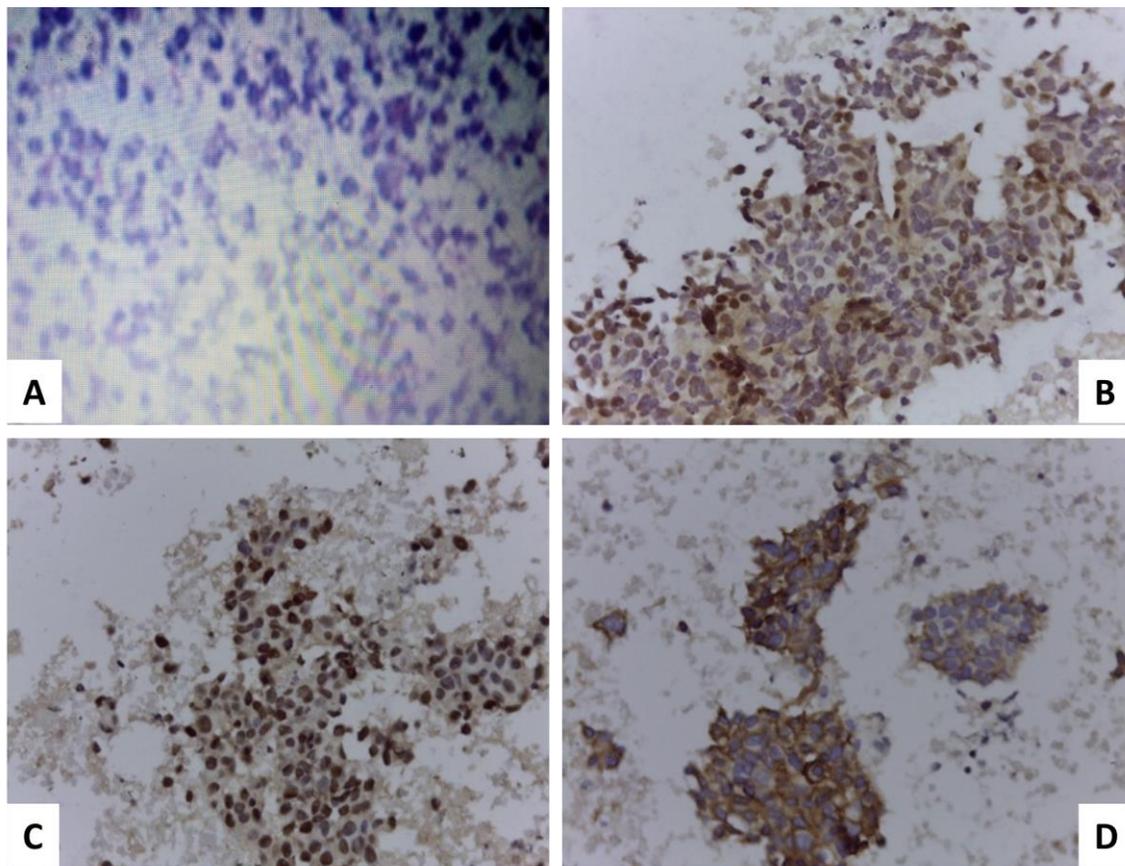
Figure 3: Photomicrograph showing immunocytochemistry stain performed on cell block preparation, slide A is negative control, B: ER positive, C: PR positive, and D: HER2 positive. X400,

DISCUSSION

The acceptance of fine needle aspiration cytology worldwide especially in developing countries because of its use as one of the components of the triple test in the management of palpable breast lesions cannot be overemphasized, its simplicity, high accuracy, cost effectiveness and least invasive method of obtaining diagnostic material has also made its acceptance possible (20). However, there are limitations, some of which include the inability to differentiate between benign and malignant lesion which may sometimes be due to considerable loss of cells during fixation and processing, paucity of specimen sampling, loss of histological architecture, mimicking of malignancy by inflammatory and degenerative cells leading to false negative and false positive diagnosis (9).

Cell block, a simple and reliable technique suitable to complement and reinforce diagnosis made from fine needle aspiration cytology of breast lesion has been advocated, and the increasing use of cell block for immunohistochemistry in the evaluation of prognostic and

predictive factors in breast cancers and its use in molecular studies has also further re-inforced this advocacy (21). For this reason, an attempt was made in our study to prepare and analyze samples from palpable breast masses using both conventional smear and cell block technique, comparing the results obtained and subsequently carrying out immunohistochemistry stain on the cell blocks that are positive for malignancy, using the IHC to further confirm the results obtained.

The Smear technique in our study shows that 10% of the outcome were inadequate and were graded as C1, 4% were bloody cells and were graded as C1 unsatisfactory. Another 4% were acellular and were graded as C1, making the total of about 18% of C1 as shown in the cumulative percent. Majority of the result shows 114 participants that is 76% to be C2 benign, with 2% showing result of C4 (Suspicious for malignancy) and 4% being cancerous in nature (C5 malignant). The Cell block technique on the order hand revealed about 74%, representing about 111 participants to be benign, 10% representing 15 participants were C1 (inadequate), while 4% were C1 (inflammatory), 6% are C3, with another 6 % found to be C5 malignant. The analysis from the cumulative frequency shows that about 6% of the results were malignant. The smear diagnosis tends to be less effective when compared with cell block diagnosis, as Cell block diagnosis was able to reveal about 6% of malignancy (C5 Malignant) when compared to the smear diagnosis which revealed 4% of malignancy, cases also reported as benign with the smear method where also found to be incorrect when Cell block was used. IHC staining for ER, PR and HER2 performed on the malignant cases

obtained from both methods revealed that cell blocks showed better architectural patterns, high cellularity, morphological features and an additional yield of malignant cells than the smear method, this is in agreement with a study carried out by (21)

This study also agrees with the study carried out by (22) that shows that cell blocks can reliably be obtained from FNAs with sufficient material of diagnostic value as well as the study carried out by (23) that also reported that cell block technique allowed them to recover minute cellular material that was useful for their immunocytochemistry (23, 24).

The contribution of cell blocks to the final cytologic diagnosis supports the view that cell blocks should be considered in all fine needle aspiration of breast specimens whenever possible. Cell blocks in this study has complemented and added to the diagnostic utility of the smears method, it has also allowed the preservation of the number of epithelial cell clusters (ECC) expected. It is reproducible and will therefore aid ancillary study such as the immunohistochemical analysis that was performed successfully without compromising the antigenic preservation and this really enhanced the diagnostic utility of cell block.

CONCLUSION
The Cell block method with biomarker gave better architectural patterns, high cellularity, morphological features and additional yield of malignant cells than the smear method, cell block is hence recommended to be considered as complementary to smear method especially where IHC is required for suspicious cases. This study thus further support the claim that cell block method

can also be used to supplement the smear technique where clinical diagnosis is suggestive of malignancy.

Conflict of interest- Non

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