Modified Plasma-Thrombin Method of Cell Block Preparation for Fine-Needle Aspiration Biopsies in Resource-Limited Settings

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ABSTRACT

Objectives: *The plasma-thrombin method is commonly used to make cell blocks from fine-needle aspiration (FNA) samples but requires centrifugation. We describe a modification to this method that does not require centrifugation for use in resource-limited settings.*

Methods: *Pooled fresh plasma is aliquoted into 2-mL Eppendorf tubes and the FNA sample directly rinsed into the plasma. Two drops of reconstituted thrombin are added and gently mixed. A cell clot is transferred to a tissue bag, fixed in formalin, and processed. This method was applied to FNA samples from 44 patients presenting to the Mbarara University of Science and Technology FNA clinic.*

Results: *The cell blocks were less cellular than the smears but contained adequate material to confirm morphologic impression or perform immunocytochemistry in 36 of 44 cases (82% adequacy rate).*

Conclusions: *The modified plasma-thrombin method is a reliable cell block preparation method that can be easily applied in resource-limited settings.*

Fine-needle aspiration (FNA) biopsies are highly advantageous in resource-constrained settings, providing a rapid and cost-effective method of obtaining diagnoses with high sensitivity and specificity in comparison to surgical biopsies.[1-3] The preparation of cell block specimens and addition of immunohistochemistry and other molecular techniques to FNA biopsy specimens allow for more-specific diagnoses to be obtained on FNA. Examples of scenarios requiring cell block and immunohistochemistry to yield specific diagnoses on FNA include the determination of site of origin of metastatic tumor,[4-7] the definite classification of poorly differentiated malignancies,[8-10] categorizing spindle cell lesions,[11-13] and the subclassification of lymphomas.[14-18] A strategy combining FNA smears with cell block and immunohistochemistry often obviates the need for surgical biopsies to obtain definite and specific diagnosis.

Aside from reaching a specific diagnosis, cell block and immunohistochemistry on FNA specimens are useful for evaluating potential responses to therapy by testing for the expression of predictive markers. Such markers include BRAF(V600E)[19] for predicting response to the BRAF inhibitor vemurafenib in melanoma, and estrogen receptor/progesterone receptor (ER/PR) and Her2,[20-26] for respectively predicting response to endocrine therapy and trastuzumab in breast carcinomas.

A variety of methods can be used to create cell blocks from FNA biopsy specimens.[27] The process, equipment, supplies, advantages, and disadvantages for these methods are summarized in **Table 1**.[28-36]

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Table 1
Advantages and Disadvantages of the Various Cell Block Preparation Techniques, Including Our Modified Plasma-Thrombin Method

Cell Block Preparation Method	Equipment/Supplies	Advantages	Disadvantages
Plasma-thrombin[28-30]	Centrifuge, Roswell Park Memorial Institute (RPMI) medium or saline, plasma, thrombin	Cheap and relatively easy to perform	Requires a centrifuge Risk for specimen loss upon decanting Potential for decreased antigenicity upon exposure to saline
HistoGel agar[31]	Centrifuge, microwave, HistoGel or agar	Good cellular preservation	Requires a centrifuge Risk for specimen loss/degeneration upon exposure to hot HistoGel or agar
Collodion bag[32]	Centrifuge, collodion, fume hood	Excellent cellular preservation	Requires a centrifuge Requires special technical knowledge (tedious technique) Stored in ether-based solvent: risk for generating ether fumes and flammable peroxidases; requires handling in a laminar flow fume hood and storage in a flame-proof cabinet
Cellient[33,34]	Centrifuge, proprietary instrument, Cytolyt (a methanol-based fixative)	Good cellular preservation	Requires a centrifuge Cost of proprietary instrument Longer time to prepare cell blocks
Dry clotting and vapor fixation techniques[35,36]	None	Cheap Does not require special equipment	Variable yield
Modified plasma-thrombin	Plasma, thrombin	Cheap Easy to perform Does not require special equipment Reliable yield: no decanting hence minimal risk for specimen loss No saline exposure hence preserved antigenicity	Cellularity is only moderate compared to the accompanying smears

Having assessed the various cell block preparation methods, the plasma-thrombin method, due to its ease of preparation, was the most likely to be adaptable to resource-limited settings, such as the Mbarara University of Science and Technology (MUST) histopathology laboratory. MUST is affiliated with Mbarara Regional Referral Hospital (MRRH), a 600-bed hospital in southwestern Uganda. MRRH is government funded through the Ministry of Health and covers Mbarara and surrounding districts, serving a population of 4,376,262 (Uganda Bureau of Statistics population census, 2002).

The MUST histopathology department is the only public histopathology laboratory in the southwestern region. The histology laboratory has a recently installed inverter battery electrical system, a tissue embedding system, a microtome, a tissue processor, and a manual slide staining and labeling operation; slides and reports are also stored in the histology laboratory. The department is staffed by three full-time histotechnicians and (usually) one anatomic pathologist. The MUST histopathology department currently performs or reviews an average of 50 FNA cytology cases per month. Referrals are predominantly for enlarged lymph nodes with suspicion of lymphomas or metastatic carcinomas, as well as palpable breast masses to distinguish benign fibroadenomas from breast carcinomas. Thyroid nodules, salivary nodules, soft tissue masses, bone marrow, and deep organ aspirations comprise the remainder of the cases. Occasionally, FNA alone is insufficient for accurately subclassifying lymphomas and soft tissue masses and immunophenotyping is needed. The MUST histopathology department has the case volume and capacity to perform manual immunohistochemistry as well as the faculty to interpret results. The MRRH clinicians are able to refine patient care based on specific pathology diagnoses such as specific lymphoma subclassification and ER/PR status in breast carcinomas. However, specific diagnoses were not always possible on FNA, as cell blocks were not routinely prepared from the FNA specimens. As the histopathology laboratory did not have a working centrifuge, microwave, or fume hood, we needed a simple, adaptable cell block preparation method.

One potential reason for lower yields associated with the plasma-thrombin method is cell pellet loss in the process of decanting after centrifugation. Another disadvantage is the possibility for decreased antigenicity (for immunohistochemistry) upon saline/water exposure.[37] We have introduced a modification of the plasma-thrombin method that does not require centrifugation and in which

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the FNA biopsy needle is directly rinsed into plasma (and hence reduces the likelihood of cell loss or altered antigenicity).

Materials and Methods

We performed a prospectively sampled method-comparison study in which cell blocks prepared via the modified plasma-thrombin method obtained from FNA biopsies of clinically suspicious masses from patients presenting to the MUST histopathology department were compared to cytologic smears for diagnostic adequacy.

Sampling and Sample Size Determination

The minimum sample size (n = 35) was determined using normal approximation for sensitivity where disease status is known,[38] according to the formula:

$$n = \frac{Z^2 P (1 - P)}{d^2}$$

where Z = critical value at 95% confidence interval (1.96), P = sensitivity or specificity (based on prior studies comparing cell block to histology, we expected approximately 90% sensitivity/specificity for this study), and d = margin of error (10%).

Procedures

FNA Biopsy

For superficial sites, a 23-gauge needle attached to a syringe is inserted into the patient's mass and agitated to obtain cells. The cells obtained are smeared on a slide and then the needle rinsed into plasma for cell block preparation. Up to three passes are performed. Aspiration of deep sites (liver) are performed percutaneously under ultrasound guidance using a 23- or 25-gauge spinal needle. Personnel performing the FNA biopsy procedure include a cytopathologist and pathology postgraduate trainees (residents). Dedicated needle passes to obtain a richer samples for cell blocks are performed in certain cases.

Cell Block Preparation

Pooled fresh plasma is obtained from the Mbarara blood bank and 0.5 mL aliquoted into 2-mL Eppendorf tubes and frozen. At the time of FNA, a tube of aliquoted fresh-frozen plasma is thawed. The FNA biopsy needle is gently rinsed in the 0.5-mL plasma aliquot, and two drops of thrombin are added and the tube gently flicked to mix in the thrombin. A clot forms immediately. The clot is immediately transferred to a tissue bag and cassette, fixed in formalin, and subjected to routine histologic processing, H&E staining, and immunohistochemistry, if necessary.

Histologic Processing

Histologic processing is performed according to the routine MUST laboratory procedure. A tissue processor takes the sample through formalin fixative, progressive percentages of alcohol to dehydrate tissues, xylene to clear the alcohol from tissues, and two changes of molten paraffin wax to remove xylene and prepare the tissues for embedding. Additional paraffin is added to the sample to embed it in a wax block using an embedding machine. The sample is then sectioned on a microtome and collected onto glass slides, which are baked in an oven and then stained using H&E (Harris hematoxylin, progressive type).

Immunohistochemistry

Immunohistochemistry is performed on cases deemed by the pathologist as requiring immunohistochemical analysis to aid diagnosis or predict response to endocrine (tamoxifen) therapy in cases of breast malignancies. Standard immunohistochemical staining protocols are followed per the MUST manual immunohistochemistry standard operating procedure. Briefly, sections are mounted on charged slides and placed in the oven overnight at 50°C to 52°C. Sections are deparaffinized and hydrated through changes of xylene, alcohol, and distilled water. Heat-induced antigen retrieval is performed in a Bio SB (Santa Barbara, CA) pressure cooker with one times citrate buffer for 15 minutes under high pressure, according to manufacturer instructions. Slides are rinsed in distilled water, sections circled with a pap pen, and slides placed in diluted Tris buffer (Dako, Carpinteria, CA). Peroxidase block (Dako EnVision+ horseradish peroxidase [HRP] system) is added to slides for 10 minutes. Slides are rinsed in Tris buffer and then incubated in primary antibodies for 40 minutes to 1 hour at room temperature. Slides are rinsed in Tris buffer and then incubated in labeled polymer HRP antimouse or antirabbit (Dako EnVision+ HRP system), depending on which primary antibody is used, for 30 minutes at room temperature. Slides are rinsed in Tris buffer and then incubated in 3,3'-diaminobenzidine (DAB) chromogen (Dako EnVision+ HRP system) for 5 minutes at room temperature. Slides are rinsed in distilled water and then counter-stained with hematoxylin, dehydrated, and mounted. The following antibodies were used: rabbit antihuman ER ready-to-use clone EP1 (Dako), mouse antihuman PR clone PgR 636 (Dako; 1:50 dilution), rabbit monoclonal MyoD1 EP212 ready-to-use (Cell Marque, Rocklin, CA), mouse monoclonal CD3 ready-to-use (Leica Biosystems, Wetzlar,

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Table 2
The Mbarara University of Science and Technology Experience With the Modified Plasma-Thrombin Cell Block Preparation Method

Site (No.)	Diagnosis on FNA (No.)	Use of Cell Blocks (No.)
Lymph node (18)	Lymphoma (15)	Subcategorize lymphoma: 3 T-cell LBL/ALL; 1 (likely B) ALL; 4 high-grade B-cell lymphomas (likely DLBCL); 1 B-cell lymphoma (likely MCL); 2 Burkitt's; 1 mature T-cell lymphoma
	Metastatic carcinoma (1)	Contained insufficient material (3)
	Acute leukemia, NOS (2)	Confirm diagnosis via IHC (1)
		Perform IHC: probable AML (1); AML (1)
Breast (16)	Ductal carcinoma (16)	Test for ER/PR expression (14)
Scalp (1)	Metastatic neuroblastoma (1)	Confirm morphologic impression (1)
Thigh (1)	Spindle cell neoplasm (1)	Contained insufficient material (1)
Abdominal wall/retroperitoneal (4)	Poorly differentiated malignancy (1)	Reach definite diagnosis: metastatic carcinoma (1)
	Rhabdomyosarcoma (2)	Confirm diagnosis via IHC (2)
	Favor seminoma (1)	Exclude lymphoma (1)
Liver (3)	Metastatic adenocarcinoma (2)	Confirm morphologic impression (2)
	Favor hepatoblastoma (1)	Contained insufficient material (1)
Supraorbital (1)	Plasmacytoma (1)	Contained insufficient material (1)
Total (44)	Smears cellular (44)	Cell blocks sufficient (36; 82%)

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; DLBCL, diffuse large B-cell lymphoma; ER, estrogen receptor; FNA, fine-needle aspiration; IHC, immunohistochemistry; LBL, lymphoblastic lymphoma; MCL, mantle cell lymphoma; NOS, not otherwise specified; PR, progesterone receptor.

Germany), mouse monoclonal CD20 ready-to-use (Leica), mouse monoclonal CD10 ready-to-use (Leica), mouse monoclonal terminal deoxynucleotidyl transferase (TdT) ready-to-use (Leica), mouse monoclonal CD45 X16/99 ready-to-use (Leica), and mouse antihuman cytokeratin AE1/AE3 ready-to-use (Dako). Immunohistochemistry results are interpreted by a pathologist based on expected staining pattern and appropriate antibody localization. Control tissues used are: lymph node (for CD3, CD20, and CD10), thymus (for TdT), gastrointestinal tract (for cytokeratin), confirmed rhabdomyosarcoma (for MyoD1), breast carcinoma with lobular and ductal features (for ER- and PR-positive control), and breast metaplastic carcinoma (for ER- and PR-negative control).

Data Analysis

The FNA smears and accompanying cell block were reviewed by a pathologist to determine if the cell block contained sufficient material to either support/confirm morphologic diagnostic impression reached on smears and/or perform immunohistochemical analysis. The adequacy of the modified plasma-thrombin method of cell block preparation was determined using the accompanying

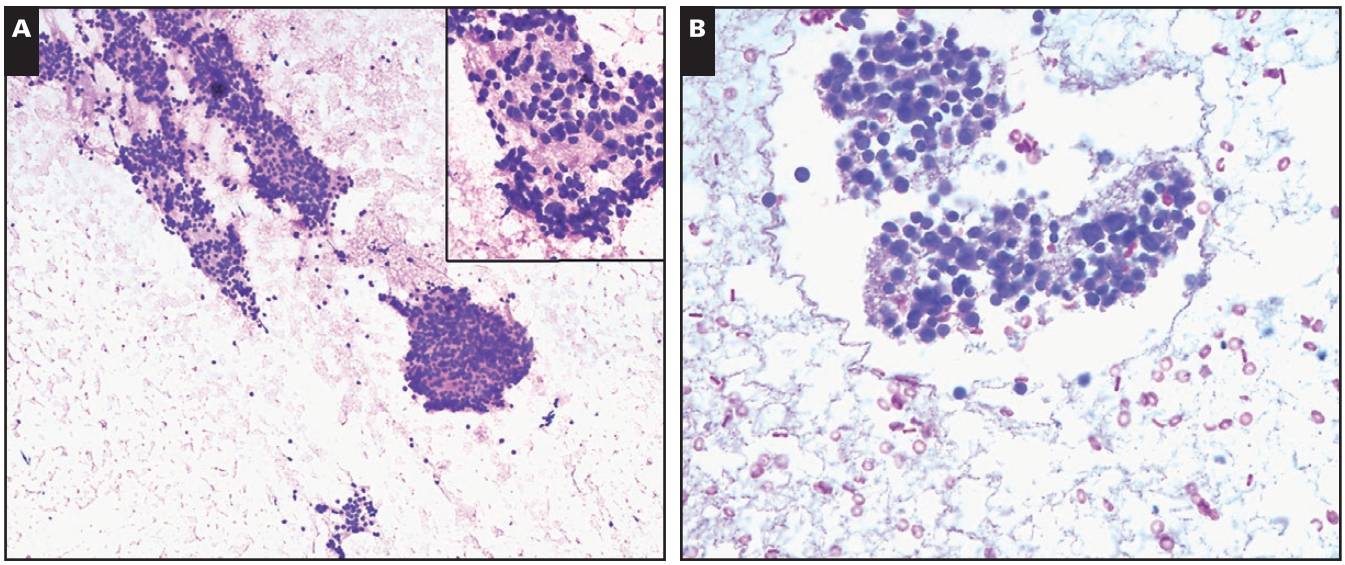


Image 1 Smears and cell block from the scalp of a 5-year-old boy with abdominal and scalp masses, clinically worrisome for metastatic neuroblastoma. The smears were cellular and comprised of a small blue cell tumor with neuropil, suggestive of neuroblastoma (**A**, ×4; inset, ×20). The cell block was less cellular than the smears but contained sufficient material to confirm smear morphologic impression (**B**, ×20).

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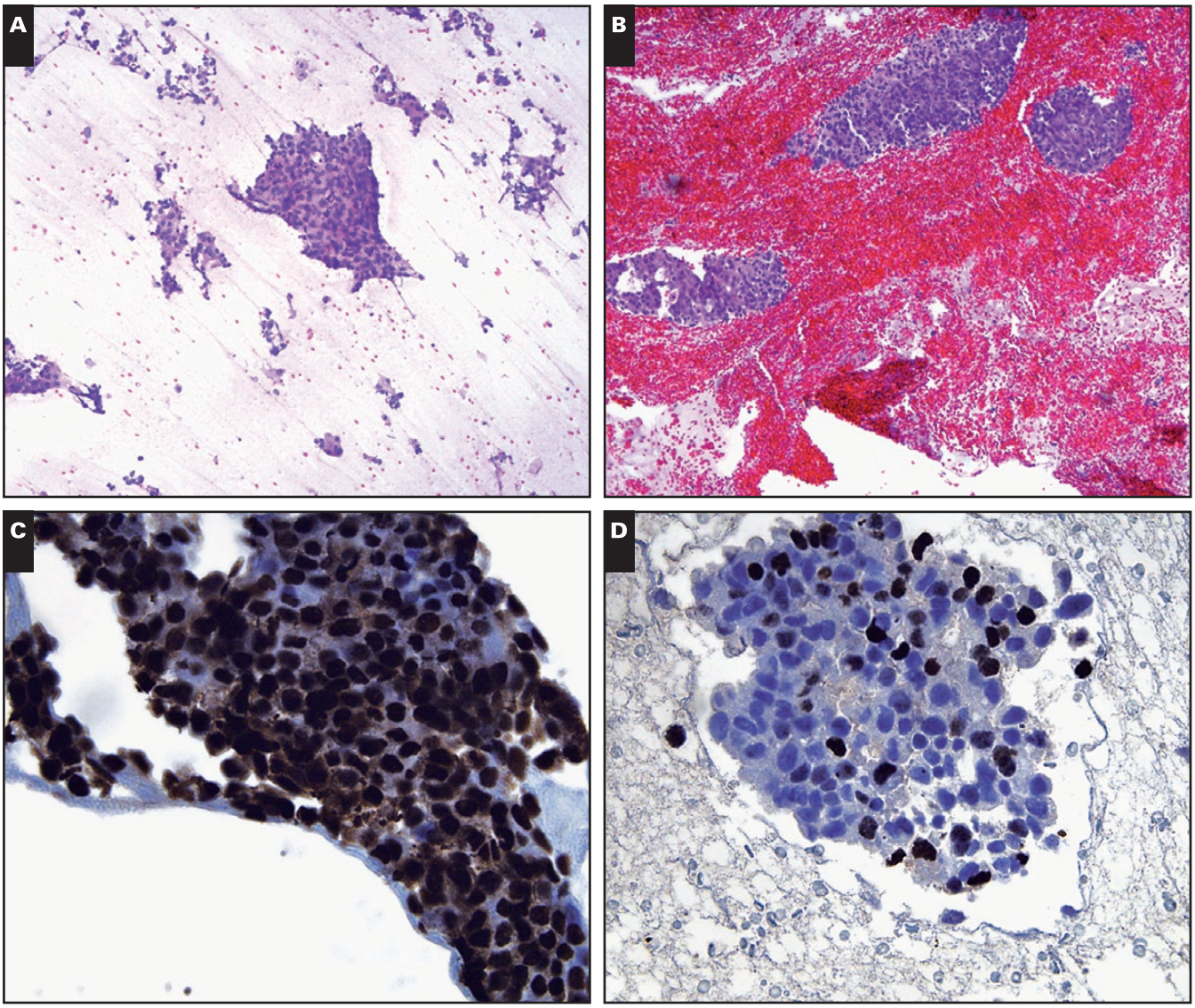


Image 2 Smear (**A**, ×4), cell block (**B**, ×4), estrogen receptor on cell block (**C**, ×20), and progesterone receptor on cell block (**D**, ×20) from fine-needle aspiration sample of breast ductal carcinoma.

smear slide as the reference/gold standard. Basic patient demographics (age and gender) as well as clinical information (location of the mass, associated symptoms, duration of symptoms, and any accompanying laboratory test abnormalities) were taken into consideration during the evaluation of smears and cell block material.

This study was approved by the Mbarara University of Science and Technology Institutional Review Board (Protocol 15/09-17).

Results

FNAs were performed on 44 masses. Aspirated sites included lymph nodes, breast, scalp, thigh, abdominal wall, retroperitoneum, liver, and supraorbital location. Diagnoses on smears were predominantly lymphoma and breast carcinoma. Cell blocks were created to confirm and to better subclassify lymphomas, perform estrogen and progesterone testing in breast carcinomas and in other tumors, and confirm diagnoses via morphologic impression or ancillary immunohistochemistry **Table 2**. In most cases, the cell blocks were less cellular than the smears but contained sufficient material to confirm morphologic impression made on smears **Image 1**, perform ER/PR testing in breast carcinomas **Image 2**, subtype lymphomas **Image 3**, or make definite diagnoses of rhabdomyosarcoma **Image 4**. Cell blocks contained insufficient material in eight of the 44 cases. The study duration was short, and therefore no data on follow-up surgical biopsies were collected.

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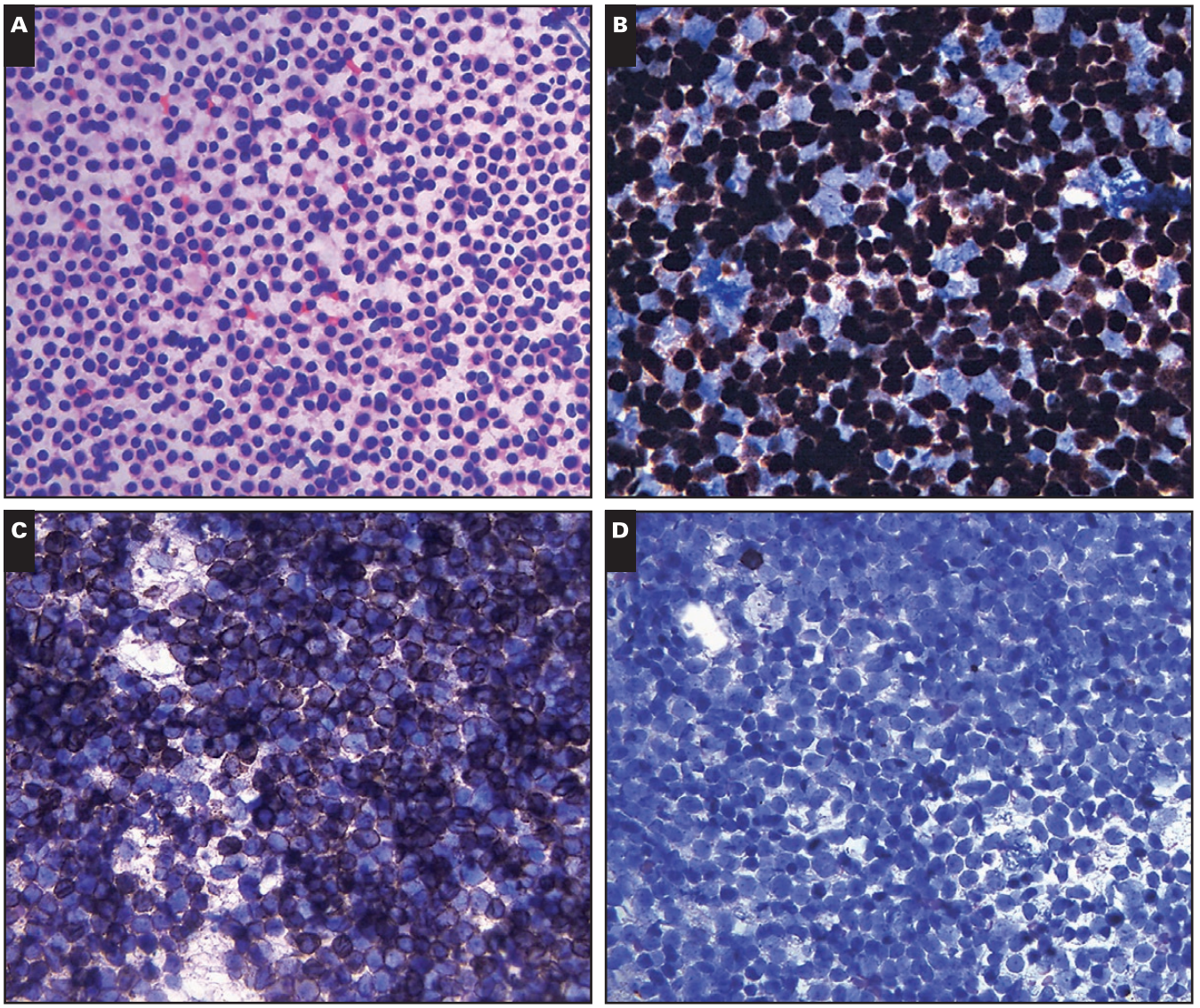


Image 3 Modified plasma-thrombin method used for lymphoma subtyping. Fine-needle aspiration of an axillary lymph node from a 25-year-old man with mediastinal, cervical, and axillary lymphadenopathy, diagnosed with non-Hodgkin lymphoma at an outside hospital; status-post 8 cycles of CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) therapy with minimal response. Based on the smear (**A**, ×10), immunohistochemical stains allowed us to make a definite diagnosis of T-lymphoblastic lymphoma (**B**, terminal deoxynucleotidyl transferase, ×10; **C**, CD3, ×10; **D**, CD20, ×10).

Discussion

Creation of cell blocks for immunohistochemistry was not routine practice at MUST histopathology laboratory. Thus, specific diagnoses such as lymphoma subtypes were not always possible on FNA. In addition, the hormonal profiles of patients' breast cancers were not routinely reported. As a result, tailored lymphoma and breast cancer therapies were not always possible. In the case of breast cancer, without knowledge of hormone status, patients with breast cancer indiscriminately received hormonal therapy with tamoxifen.

Treating patients with hormone receptor-negative tumors with tamoxifen unnecessarily exposes them to potential adverse side effects, including increased risk for endometrial cancer and venous thromboembolic events (eg, deep venous thrombosis, pulmonary embolism, and stroke) as well as reduced efficacy of human immunodeficiency virus (HIV) therapies through drug interactions.[39]

Barriers to cell block creation in resource-limited settings such as ours include requirement for specialized technical know-how and equipment expense encountered in some cell block preparation methods. We have introduced a cell block preparation method that requires limited specialized technical knowledge and no special equipment into the MUST histopathology lab.

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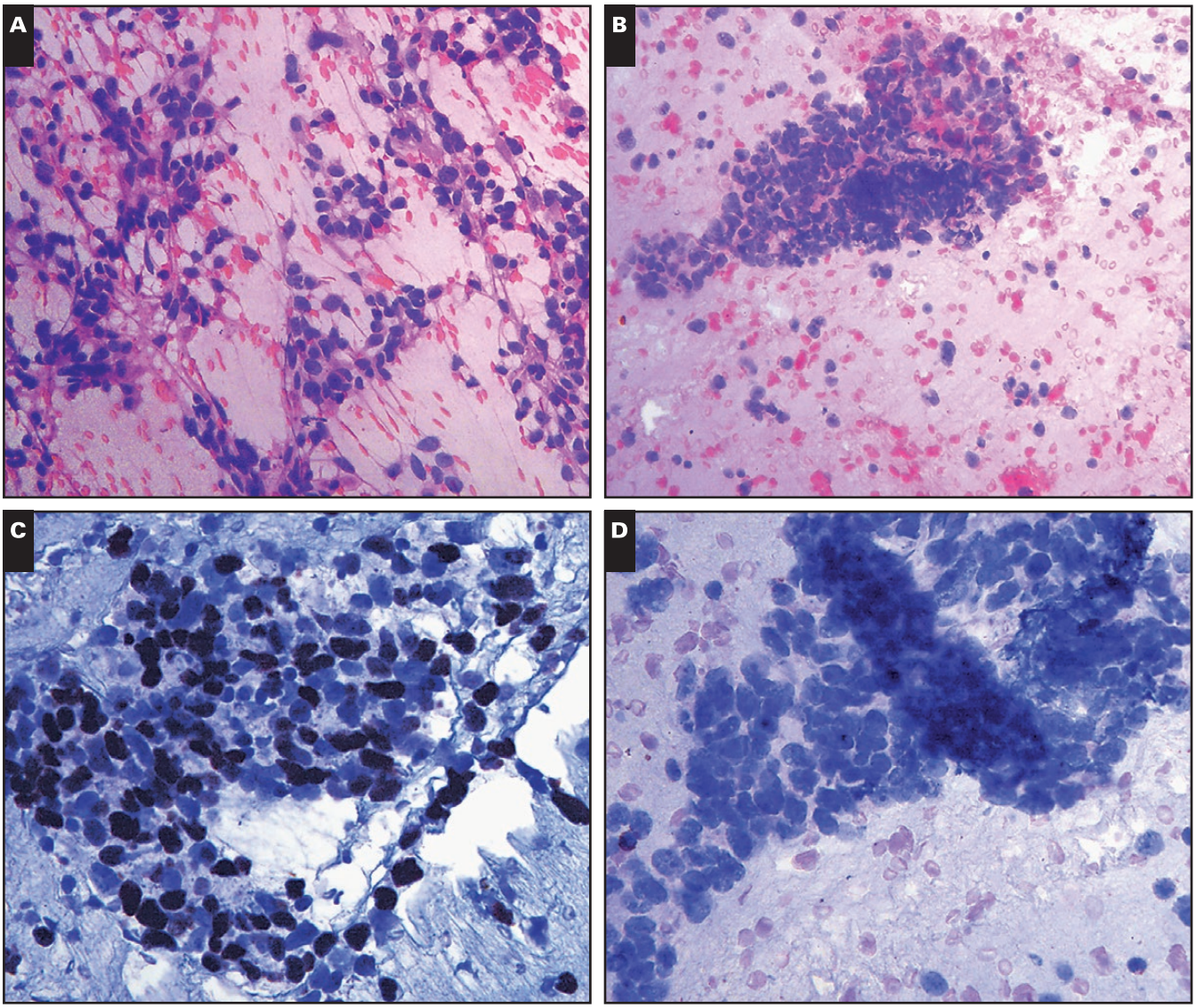


Image 4 Modified plasma-thrombin method used to confirm the diagnosis of rhabdomyosarcoma. Smear (**A**, ×10) and cell blocks (**B**, H&E, ×10; **C**, MyoD1, ×10; **D**, CD45, ×10).

Using this method, we could better subtype lymphomas using a limited panel of antibodies (CD3, CD20, CD10, and TdT) in 13 out of 15 cases and perform ER/PR testing to guide tamoxifen therapy in 14 out of 16 cases. Overall, our method yielded adequately cellular specimens to confirm morphologic impression or perform immunohistochemical testing in 82% of our cases.

Factors that influenced specimen adequacy included the volume of plasma and the ratio of plasma:thrombin used. We had initially started our protocol using 1 mL of plasma to two drops of recombinant thrombin but observed that the higher volume of plasma was associated with specimen loss, as only a portion of the sample clotted. In our experience, 0.5 mL of plasma was sufficient volume for rinsing the FNA needle and allowing the entire specimen to form a clot upon the addition of two drops of recombinant thrombin. The experience of the aspirator influenced the adequacy of the cell block. More cellular cell blocks were obtained when FNAs were performed by personnel (the faculty pathologist and the senior trainees) with experience rinsing the FNA needle in a medium. The cellularity of cell blocks increased as the junior trainees gained practice. The performance of a dedicated pass increased the yield of the cell blocks.

Our method allows for a cost-effective immunophenotyping methodology. The technique is now used routinely in cytopathologic practice at MUST. The introduction of this method has resulted in rational tamoxifen use as well as better-targeted lymphoma therapy. This method allows MUST patients to have access to immunophenotyping for lymphoma subclassification as well as preoperative receptor testing of breast cancers, the current standard of

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lymphoma and breast cancer diagnostic care in developed nations. We encourage adoption of this method in other resource-limited settings, as it is simple, cost effective, and clinically relevant.

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