

**A COMPARISON OF MODIFIED AND STANDARD PAP STAINING  
METHODS IN THE ASSESSMENT OF CERVICAL SMEARS AT  
KENYATTA NATIONAL HOSPITAL**

**BY**

**GACHIE ROSE NYAMBURA**

**BSC (JKUAT), HND & DIPLOMA MLS (KMTC)**

**H568/71016/2009**

**A DISSERTATION SUBMITTED IN PARTIAL FULLFILMENT FOR  
THE DEGREE OF MASTERS OF SCIENCE IN CLINICAL  
CYTOLOGY IN THE UNIVERSITY OF NAIROBI**

**2010**

University of NAIROBI Library



0380118 0

**UNIVERSITY OF NAIROBI  
MEDICAL LIBRARY**

**USE IN THE LIBRARY ONLY**

## DECLARATION

I hereby solemnly declare that the work contained in this dissertation is my original data and has not, to the best of my knowledge been presented at any other institution of higher learning

Signature  Date 22/3/2011

Rose. N. Gachie, postgraduate student in Masters Of Science Department of human pathology, college of health sciences, school of Medicine University of Nairobi.

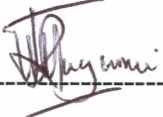
## Supervisors' declaration

This dissertation has been submitted for examination with our approval as university supervisors

Dr. Muchiri Lucy, senior lecturer, Department of Human Pathology, College of health sciences, School of Medicine, University of Nairobi

Signature  Date 22/3/11

Dr. Ndungu J.R, Lecturer, Department of Human Pathology, College of health sciences, University of Nairobi School of medicine

Signature  Date 25/03/2011

## **DEDICATION**

I dedicate this project to my beloved husband Fredrick G Mbugua and my dear son George Mbugua whose moral support and understanding made my study a success.

More dedication goes to all those fighting with cervical cancer.

## **ACKNOWLEDGEMENT**

I thank God for being my strength in difficult moments.

My gratitude is to all family members and friends for their moral support during this period of study.

Special gratitude is to my supervisors Dr. Lucy Muchiri and Dr. J R Ndungu for their tireless efforts in correcting, supervision and encouragement throughout the entire period of my study while I struggled to meet the dead line.

Sincere appreciation too to Dr. Muchiri and Dr. Gontier for their assistance with the supply of cytospin kits when I was almost grounded.

Special thanks to all the lecturers in Human Pathology Department for their motivation, advice and support during conceptualization of this study. I'm mostly indebted to Professor C. Kingondu for her motherly support, thank you.

Sincere appreciation to all my colleagues, whom in one way or another contributed to completion of this study. Special acknowledgement is to F. Tawuo and Esther Njagi for their assistance in the data collection procedures.

Most grateful too is to the histopathology department UON and KNH for allowing me to use their facilities for my study. Credits go to Wangechi and Kairu for their time and effort.

Last but not least is my appreciation to the staffs of Gynecology clinic 18 and FP clinic 66 for their hard work in the sample collection.

# TABLE OF CONTENTS

TILLE.....	i
DECLARATION .....	ii
DEDICATION .....	iii
ACKNOWLEDGEMENT .....	iv
TABLE OF CONTENTS.....	v
LIST OF ABBREVIATIONS.....	vii
LIST OF TABLES.....	viii
LIST OF FIGURES .....	ix
LIST OF PHOTOMICROGRAPHS.....	x
LIST OF APPENDICES.....	xi
ABSTRACT.....	xii
CHAPTER ONE: INTRODUCTION/BACKGROUND.....	1
CHAPTER TWO: LITERATURE REVIEW .....	3
2.1 Epidemiology .....	3
2.2 Pathogenesis.....	3
2.3 Cervical cancer Screening.....	3
2.3 .1 Liquid-based, thin-layer cytology (TP).....	5
2.3.2 Human papilloma virus (HPV) typing .....	5
2.3.3 Visual inspection with acetic acid (VIA).....	5
2.4 Pap stain modifications .....	6
2.5 Modified Papanicoloau stain.....	10
2.6 Justification.....	12
2.7 Research question .....	13
2.8 Objectives.....	13
2.8.2 Broad objective .....	13
2.8.3 Specific objectives .....	13
CHAPTER THREE: METHODOLOGY .....	14
3.1 Study design.....	14

3.2 Study area.....	14
3.3 Study population .....	14
3.4 Inclusion criteria .....	14
3.5 Exclusion criteria .....	14
3.6 Sample size determination .....	15
3.7 Sampling method .....	16
3.8 Recruitment and consenting procedure.....	16
3.9 Data collection procedures.....	16
3.10 Sample collection.....	16
3.11 Laboratory procedures .....	17
3.11.1 Staining .....	18
3.11.2 Microscopy.....	19
3.12 Variables .....	20
3.12.1 Independent variables.....	20
3.12.2 Dependent variables.....	20
3.13 Materials/Equipment.....	20
3.13.1 Equipment.....	20
3.13.2 Supplies.....	20
3.13.3 Personnel.....	20
3.14 Training procedures .....	21
3.15 Quality assurance procedures.....	21
3.16 Data collection instrument .....	21
3.17 Ethical considerations .....	22
3.18 Data management and statistical analysis plan.....	23
3.19 Study limitations .....	23
CHAPTER FOUR: RESULTS .....	24
CHAPTER FIVE: DISCUSSION, CONCLUSION AND RECOMMEDATIONS.....	41
REFERENCES.....	48
APPENDICES .....	51

## LIST OF ABBREVIATIONS

AGC-NOS	-	Atypical Glandular Cells, Not Otherwise Specified
ASC-H	-	Atypical Squamous Cells—Cannot Exclude High-Grade Lesion
ASC-US	-	Atypical Squamous Cells—Undetermined Significance
DPX	-	Distrene Dibutylphalate Xylene
EA	-	Eosin Azure
ECA	-	Epithelial Cell Abnormality
FNA	-	Fine Needle Aspiration
HPV	-	Human Papilloma virus
HSIL	-	High-Grade Squamous Intraepithelial Lesion
KNH	-	Kenyatta National Hospital
Lab	-	Laboratory
LSIL	-	Low-Grade Squamous Intraepithelial Lesion
MP	-	Modified Papanicolaou
MUFP	-	Modified Ultra Fine Needle Aspiration Papanicolaou
NILM	-	Negative for Intraepithelial Lesion or Malignancy
OG	-	Orange G
PAP	-	Papanicolaou Smear/Test/Conventional Papanicolaou
REAP	-	Rapid, Economic, Acetic Acid Papanicolaou Stain
SCC	-	Squamous Cell Carcinoma
SP	-	Standard Papanicolaou
TBS	-	The Bethesda System
UON	-	University of Nairobi

## **LIST OF TABLES**

TABLE 1: RISK FACTORS FOR CERVICAL CANCER OF THE STUDY PARTICIPANTS, .....	28
TABLE 2: QUANTIFIED COST AND TIME FOR THE TWO METHODS .....	31
TABLE 3: CYTOMORPHOLOGIC FEATURES .....	32



## LIST OF FIGURES

FIGURE 1: AGE DISTRIBUTION OF THE WOMEN SCREENED FOR CERVICAL CANCER.....	24
FIGURE 2: NUMBER OF SEXUAL PARTNERS IN THE LAST SIX MONTHS PRIOR TO THE STUDY	25
FIGURE 3: AGE AT FIRST SEXUAL INTERCOURSE.....	26
FIGURE 4: CERVICAL APPEARANCE ON GROSS EXAMINATION. ....	27
FIGURE 5: STAINING OF THE CELL BORDERS .....	33
FIGURE 6: CYTOPLASMIC STAINING .....	34
FIGURE 7: STAINING OF NUCLEAR BORDERS.....	35
FIGURE 8: CHROMATIN STAINING (APPEARANCE) .....	36
FIGURE 9: PAP SMEAR RESULTS (CYTODIAGNOSIS).....	40

## LIST OF PHOTOMICROGRAPHS

1. NORMAL PAP SMEAR.....	37
2. CLUE CELLS.....	38
3. HIGH GRADE INTRAEPITHELIAL LESION .....	38

**LIST OF APPENDICES**

APPENDIX I: CONSENT FORM ..... 51

APPENDIX II: QUESTIONNAIRE ..... 54

APPENDIX III: RESULTS FORM..... 56

APPENDIX IV: DUMMY TABLES OF THE RESULTS..... 57

TABLE (1): RESULTS PER SMEAR ..... 57

APPENDIX V: WORKING PRINCIPLE OF A SHANDON CYTOSPIN 4..... 60

APPENDIX VI: PAP SMEAR COLLECTING PROCEDURE..... 61

APPENDIX VII: STANDARD PAPANICOLOAU STAINING METHOD ..... 62

APPENDIX VIII: MODIFIED PAP STAINING METHOD ..... 63

APPENDIX IX: THE BETHESDA SYSTEM 2001 ..... 64

## **ABSTRACT**

Invasive carcinoma of the cervix is one of the most common cancers in the world. Cervical cancer is preventable by screening methods. The traditional screening method is the Pap smear. As practiced conventionally Pap stain is expensive utilizing a considerable amount of alcohol and consumes a lot of time. Several Pap modifications have been made to reduce both alcohol use and time and they have proved to be cheaper and timely and reveals improved morphological staining quality with no diagnostic compromise.

**Objective:** To compare modified and standard Papanicolaou (Pap) staining methods in the assessment of the cervicovaginal smears in respect to cytomorphological features, cytodagnosis, reagents, time and cost.

### **Design**

A descriptive cross sectional study

### **Study area**

Gynecology clinic 18, FP clinic 66 and cytology laboratory at KNH

### **Study population**

Women attending the study areas at KNH and who were eligible for a pap smear.

**Methods:** One hundred and sixty two coded paired cervical smears were prepared by liquid based method. One set of smears was stained by the standard Pap staining protocol (SP) and the other by the modified Pap protocol (MP) in which alcohol was replaced by 0.5% acetic acid. The smears were blinded and examined by the investigator and then the pathologists and decoded. Each pair of smears was compared and the two protocols were analysed for staining quality and cytodignoses statistically by use of Chi square.

**Results:** The study showed that the time taken to stain each bunch Pap smear slides in the modified Pap method is  $3 \pm 0.5$  minutes while in the standard Pap method the time taken was  $20 \pm 0.5$  minutes for the same number of slides. In MP the cost of alcohol used per smear was Ksh 18.50 and Ksh 123.45 per smear in the SP.

The standard Pap method had 14 (8.8%) unsatisfactory cytoplasmic staining, 102 (64.2%) were satisfactory and 43 (27.0%) were excellent while Modified Pap method had 10 (6.3%) unsatisfactory cytoplasmic staining, 53 (33.3%) satisfactory and 96 (60.4%) were excellent. The nuclear staining was hazy in 79 (49.7%) and distinct in 80 (50.33%) in the standard Pap, while in the modified Pap, the nuclear staining was hazy in 54 (34%) and distinct in 105 (66%). The results for the cytodiagnosis concurred in both the modified and standard Pap methods.

**Conclusions:**

The modified Pap staining method is simple, low cost and better in the staining of the cervical smears and can therefore be used as alternative to the standard Pap method in the screening for cervical cancer.

**Recommendations:**

Modified Pap method should be introduced for the screening of cervical cancer in low resource settings.

## CHAPTER ONE: INTRODUCTION/BACKGROUND

Invasive carcinoma of the cervix is the second commonest cancer in women after breast in the world. Among developing countries, it is the commonest cancer and the leading cause of cancer deaths in women between the ages of 35 and 45 years. It is also the leading virally induced cancer worldwide. <sup>(1)</sup> It is preceded, almost without exception, by precancerous lesions that develop over several years. Nevertheless, only 10% of these precancerous lesions develop into invasive cancers while others regress. <sup>(2)</sup>

Cervical cancer is preventable, and screening methods exist to detect it at a precancerous state. Cytology-based Pap smear is one such reliable tool. <sup>(3)</sup> The Pap smear is the standard screening tool used to test for the presence of abnormal cells that could become neoplastic. A regular Pap smear provides an opportunity to detect pre-cancerous cells in the cervix. <sup>(4)</sup> The routine Pap test has never been examined in a randomized controlled trial; however, a large body of consistent observational data supports its effectiveness in reducing mortality and morbidity from cervical cancer. <sup>(5)</sup> The high mortality rate from cervical cancer in women of child-bearing age may be one of the most important social problems in developing countries. <sup>(6)</sup>

Despite its demonstrated efficacy, the Pap test is widely available only in those countries with a high degree of organization, a relatively high disposable income, and a relatively well organized infrastructure and health care system. <sup>(1)</sup> To be successful, a screening program must be directed at a suitable disease with a suitable screening test defined by simplicity, acceptability to patients, low cost, and high validity. <sup>(8)</sup>

As practiced conventionally, the Pap staining procedure is expensive. It utilizes a considerable amount of alcohol and takes about 20 minutes. In addition, the alcohol and xylene require safety storage measures and costly disposal. These deterrents often jeopardize successful cervical cancer screening in resource-limited settings.

It is on the light of the above that Pap modifications have been established to reduce the use of alcohol and the staining time in order to improve the cervical cancer screening.

## **CHAPTER TWO: LITERATURE REVIEW**

### **2.1 Epidemiology**

Cervical cancer is a major cause of death worldwide, with approximately 490 000 women diagnosed annually with invasive cervical cancer and accounts for over 230 000 deaths annually. The majority of cases (80%) occur in developing countries where it is frequently the second most common cancer. <sup>(9)</sup> Epidemiological data show that annual screening reduces the mortality by 70% and the probability of developing invasive carcinoma is reduced by over 95%. <sup>(5, 10)</sup>

### **2.2 Pathogenesis**

The pathogenesis of cervical neoplasia and cervical cancer is related to HPV based on epidemiological, virological, and experimental evidence. This was suggested by Zur Hausen in the mid-1970's. <sup>(11)</sup> Infection with HPV alone may not be sufficient for development of cervical intraepithelial neoplasia and the ability of the immune response to resist changes plays an important role in development of cervical carcinoma. <sup>(12)</sup>

### **2.3 Cervical cancer Screening**

In virtually all population-based screening programs to date, the standard screening method has been the Pap smear. <sup>(1)</sup> The Papanicolaou test/smear is a screening test used to detect premalignant and malignant (cancerous) processes in the ectocervix. <sup>(6)</sup> Cervical cytology (Pap smear) was introduced by George Papanicolaou into clinical practice in 1940. The four main steps of the Pap staining method are:

1. Fixation (15 minutes).



2. Nuclear staining with hematoxylin (4 minutes).
3. Cytoplasmic staining with counter stains (EA, OG) (6 minutes).
4. Clearing (3 minutes).

The Pap stain results in well-stained nuclear chromatin, differential cytoplasmic counter staining and cytoplasmic transparency. However the intensity of nuclear and cytoplasmic staining and the colour of cytoplasm are largely a matter of personal preferences. <sup>(4)</sup>

The Papanicolaou method uses a standard nuclear stain, hematoxylin, and two cytoplasmic counter stains, OG-6 and EA. The value of this method is in the transparency of the cytoplasm, which allows the examiner to clearly visualize cellular morphology. Either a progressive or regressive technique may be used for nuclear staining. Maintenance of consistently good staining requires that the stains be filtered and changed on a regular schedule, determined either by the number of slides processed or the length of time elapsed since stains were last changed. <sup>(5)</sup>

The conventional/standard Papanicolaou smear is associated with variable false positive and false negative rates, difficulties with interpretation and high unsatisfactory and suboptimal rates with consequent low reproducibility. New techniques such as thin preparations are being developed, based on preservation of the collected material in a stabilizing solution and slide preparation in the laboratory to reduce these errors. <sup>(6)</sup>

The accuracy of this important screening tool also remains controversial, with several large meta-analyses suggesting that both the sensitivity and specificity of cervical cytology is relatively low (30% to 87% sensitivity, 86% to 100% specificity).<sup>(13)</sup>

Newer methods have been introduced to improve on the cervical cancer screening and they include:

### **2.3.1 Liquid-based, thin-layer cytology (TP)**

Liquid based-cytology (LBC) was introduced in the mid-1990s. The purported advantages of LBC include a possible increase in the detection of high-grade cervical intraepithelial neoplasia, a reduction in the number of unsatisfactory and 'satisfactory but limited by' specimens, and providing residual cellular material for subsequent molecular testing (e.g. testing for 'high-risk' types of hpv DNA).<sup>(13)</sup>

### **2.3.2 Human papilloma virus (HPV) typing**

HPV DNA testing, rather than cytological testing, is recommended for cervical cancer screening in some resource-poor areas.<sup>(9)</sup> HPV testing combined with cytology is a reasonable approach in elderly women in order to increase the screening interval to 3-5 years.<sup>(8)</sup>

### **2.3.3 Visual inspection with acetic acid (VIA)**

Visual Inspection with Acetic Acid (VIA) and Visual Inspection with Lugol's Iodine (VILI) are two modifications of a direct visual assessment of the cervix.<sup>(14)</sup> The VIA test is relatively simple, easy to administer and inexpensive, and it relies on little

infrastructure. It requires simple vinegar and the eye of a trained health-care provider to spot abnormal lesions. Both VIA and HPV vaccine and typing methods are most effective if they are combined with the Papanicolaou method. <sup>(15)</sup>

## **2.4 Pap stain modifications**

Papanicolaou stain has undergone various modifications in different laboratories. <sup>(16)</sup> The original modifications of pap stain (1942) were published by Dr. Papanicolaou in 1954 and 1960 and Papanicolaou described his formulations 3 times between 1942 and 1960. He not only significantly changed the OG and EA dye formulations in 1954 and 1960, but he also never described the compositions in quantitatively reproducible terms. Since commercially available OG and EA solutions are so variable in composition, the general rule is less time in OG and more time in EA. Consequently, all cytotechnology programs teach a different Pap stain, all vendors sell different formulations, and all laboratories have their own staining protocols. <sup>(17)</sup>

Many Pap stain short methods which were generally directed to fine needle aspirated (FNA) specimens and to the conventional cervico-vaginal smears have been sought.

One such shorter method which sought to reduce the overall time was ultrafast pap modification stain for various body organs developed by Pandit et al. <sup>(18)</sup>

In this modification; Gill's haematoxylin, EA-36, and isopropanol were used instead of Richard-Allan haematoxylin, cytochrome, and ethanol, respectively. Diagnosis made by Modified ultra fast pap (MUFP) stain was compared with standard Pap stain.

In this study, the quality of MUFP staining was evaluated on four parameters such as smear background, overall staining pattern, cell morphology, and nuclear characteristics. In most of the cases, the maximum score were obtained for all the four parameters and it was observed that MUFP stained smears had clear RBC free background, crisp nuclear chromatin, well-stained nucleoli, and transparent cytoplasm. The diagnosis made was correct except in three cases of metastatic squamous-cell carcinoma. It was concluded that MUFP stain is useful for rapid diagnosis by FNAC, but is not useful for squamous-cell lesions.

A modified rapid papanicolaou stain for imprint smear was done as intra operative cytology. <sup>(19)</sup> Hematoxylin was pre-heated to facilitate nuclear staining. The method was also not suitable for squamous cell lesions and therefore was not useful for cervical smears.

Another Pap modification to reduce the staining time was done specifically in connection with fine needle aspiration (FNA) specimens by Yang and Alvarez. <sup>(20)</sup> This pap staining method was further modified to apply to cervico-vaginal smears. <sup>(21)</sup> The Yang et al modification of the Yang-Alvarez FNA staining method required 12 separate post-fixing, pre-coverslip steps, and also required more than 15 minutes, all in disparate sequence times ranging from a 6 dip step taking about 6 seconds to a cytostain staining step taking upwards of 5 minutes. Again this method was not laboratory technician friendly in that it invited errors because of the disparate dip sequencing as well as in the number of steps, and difficult in the use of automatic staining machines.

Recent pap modification studies have been done in order to reduce time, alcohol use and improve the staining quality. One such study was done by Gupta et al.,<sup>(22)</sup> in modified Papanicolaou staining protocol with minimum alcohol use. In this protocol, alcohol was replaced by 1% acetic acid in all steps except during the initial fixation and prior to mounting. Also one alcohol based counter-stain orange G (OG) omitted. The procedure in the MP was simplified by following 10 uniform dips in each reagent at each step. The staining quality in the modified protocol was comparable to the standard protocol followed in the laboratory and there were no statistically significant differences in the two protocols. The nuclear and cytoplasmic features were comparable to the CP. Cytoplasmic transparency was maintained in the MP. The diagnoses agreed in all cases and there was no compromise in interpreting the smears. With MP it took only 3–4 minutes to stain a batch of 50 slides in contrast to the 20 minutes taken by CP. The revised protocol resulted in saving large quantities of alcohol, requiring only one-seventh of the amount of alcohol compared with the CP.

A similar study to reduce staining time and reduce alcohol use was done by Akinremi et al.,<sup>(23)</sup> in reduced alcohol use in the staining of Pap smears: a satisfactory, low cost protocol for cervical cancer screening. The amount of alcohol consumed in the procedure was reduced drastically by (1) using only one modified cytoplasmic counterstain (EA type), thereby (2) reducing the number of alcohol rinses by over half. Orange-G dye was omitted from the procedure. The resultant effect of the modified staining protocol was

quite satisfactory; nuclear details were sharp and crisp, while the cytoplasm contained transparent differential staining with blue-green and pink.

Roy et al.,<sup>(16)</sup> did another study aimed at minimizing alcohol use and time in a procedure known as rapid economic acetic acid pap (REAP). Alcohol was replaced by 1% acetic acid in all the steps except during fixation and methanol used prior to mounting instead of alcohol. *Orange G counter-stain was included in the procedure unlike the above cases.* The procedure in the REAP was simplified by following 10 uniform dips in each reagent at each step. The REAP costs about 25% of total cost of standard Papanicolaou stain therefore it was cost-effective for mass screening programme. In the REAP protocol 110 pairs of smears were used to compare the two methods. The cytoplasmic & nuclear staining was optimal in 100 & 105 smears respectively while 90 and 100 were optimal in the standard pap respectively thus making the modified pap better. The results for the cytodiagnosis concurred in both the modified and standard Pap methods and there was no statistically significant difference. The method was also very rapid and suitable for the screening of cervical smears.

Another study known as environmental friendly, economic and effective pap was done by Gill.<sup>(17)</sup> This was primarily to reduce alcohol and xylene use. In the study Post-OG/EA 95% ethanol baths were replaced by 0.5% acetic acid (HOAc), and Scott's bluing agent was replaced by tap water. Water-scavenging aluminosilicate beads were added to xylene that was filtered daily and reused indefinitely. This procedure yielded high quality, reproducible staining results while saving money by eliminating chemically defined

bluing agents (e.g., Scott's tap water substitute), consuming less alcohol, reusing xylene indefinitely, and reducing hazardous waste disposal costs.

## **2.5 Modified Papanicolaou stain**

Modified Pap method was intended to reduce the staining time and the cost of the Pap staining. Many modifications have demonstrated reduction in cost and time by reducing the amount of alcohol used in the procedures and reducing the staining steps without compromising the quality or the cytodiagnosis of the smear.

The study adopted the modification by Gupta et al.,<sup>(22)</sup> in the modified Papanicolaou protocol with minimum alcohol use. The method was selected for its simplicity with much staining time and much alcohol reduction. In this procedure 95% alcohol is replaced by 1% acetic acid except before the staining and before the mounting of the smears. The procedure was simplified by following 10 uniform dips in each reagent at each step. Orange G counter staining which consumes a lot of alcohol during its preparation was also omitted from the procedure reducing the use of alcohol further.

The staining quality in the modified protocol was comparable to the standard protocol and there were no statistically significant differences in the two protocols. The nuclear and cytoplasmic features were comparable to the SP. The diagnoses agreed in all cases and there was no compromise in interpreting the smears. With MP it took only 3–4 minutes to stain a batch of 50 slides in contrast to the 20 minutes taken by SP. The

## 2.6 Justification

Although there is overwhelming evidence that cervical cancer today is almost totally preventable to a large extent through screening and treatment of premalignant lesions, the screening programs on account of cost and lack of a systematic cervical cancer screening program in Kenya appears to be a factor in the high incidence of progressive disease. <sup>(7)</sup>

The standard diagnostic method of the cervical neoplasm as in any other neoplasm is the biopsy. However, it is expensive to use in developing countries and screening methods have been therefore utilized in those countries with poor resources.

Standard Pap smear has been the most successful cancer screening test in history since its introduction in the 1950s. <sup>(4)</sup> As practiced conventionally standard Pap stain use a substantial quantity of alcohol which hinders its use as a mass screening tool in low-resource settings. It is also a complex procedure taking a long time to complete the staining. <sup>(22)</sup>

Certain Pap staining protocols that conserve alcohol have also been published over the last decade in other countries such as India and have shown drastic reduction in the cost of Pap staining. However, none of the studies have been conducted or adopted in our local settings and it is on this light that the study was undertaken.



## **2.7 Research question**

Can modified Papanicolaou staining method be used as an alternative to standard Pap staining method in the assessment of cervical smears?

## **2.8 Objectives**

### **2.8.2 Broad objective**

To compare modified and standard Pap staining methods in the assessment of cervical smears.

### **2.8.3 Specific objectives**

1. To compare the standard and modified pap smears with respect to reagents, cost and time
2. To compare the cytomorphological features of the cells in modified and standard Pap staining methods in the assessment of cervical smears.
3. To compare the cytodiagnostic accuracy between modified Pap and the standard Pap staining methods in the assessment of cervical smears.

## **CHAPTER THREE: METHODOLOGY**

### **3.1 Study design**

This is a descriptive cross sectional study.

### **3.2 Study area**

The study was conducted at Kenyatta National Hospital (KNH). The data and sample collection was done at the gynecology and family planning clinics while the staining was done in the cytology laboratory in the hospital.

Kenyatta National Hospital is the largest hospital in Kenya and it is both a referral and a teaching hospital. It is located in the capital city, Nairobi. It essentially serves as a provincial and district hospital for the city of Nairobi.

### **3.3 Study population**

All women of 18-49 years attending Gynecology clinic number 18 and Family Planning clinic number 66 at Kenyatta National Hospital and who met the eligibility criteria.

### **3.4 Inclusion criteria**

Those included were adult women

- 18-49 years old.
- Attending Gynecology clinic number 18 and Family Planning clinic 66 at KNH.
- Who consented to participate in the study
- Had not had total hysterectomy.

### **3.5 Exclusion criteria**

All women who had a total hysterectomy

### 3.6 Sample size determination

The standard statistical approach to determination of sample size for a descriptive cross-sectional study such as this one requires specification of an estimate of the proportion (prevalence) of cervical neoplasia in patients to be estimated, the desired level of confidence desired for proportion estimate and the tolerance error margin or width of confidence interval (a measure of precision of the estimate), so that the necessary sample size is then calculable for a given precision level. A study done by Termmerman et al in Kenya indicates a prevalence of 12% which was used in this study. <sup>(24)</sup>

Sample size formula below was then used to estimate the sample size.

$$n = Z^2 \frac{P(1-P)}{D^2} \quad (25)$$

Where  $n$  = was the required sample size,

$p$  = expected prevalence or proportion among patients with the cervix neoplasia.

$D$  = degree of precision or a tolerance error margin or width of the confidence interval (a measure of precision of the estimate which ranges from 20% - 1%).

$Z$  = statistic for a level of confidence or was the normal distribution critical value of  $\alpha/2$  in each tail. For a 95% confidence,  $Z = 1.96$

For this study 95% level of confidence and an error of margin of  $\pm 5$  was considered acceptable.

Therefore  $Z = 1.96^2 (.12) (.88) / .05^2 = 162.27$ .

A sample size of 162 was recruited.

### **3.7 Sampling method**

A convenient sampling method was used.

Every woman who visited the study areas mentioned above was invited to participate and all those who met the eligibility criteria were included in the study until the desired sample size of 162 was achieved.

### **3.8 Recruitment and consenting procedure**

The investigator visited the study areas (clinics) and introduced herself to the staff who in turn introduced her to the potential clients. The investigator explained in details about the study including, the purpose of the study, ethical issues, the procedure, risks and benefits of the study. Upon agreeing to participate, the participant was given a consent form to sign as explained in the Appendix I.

### **3.9 Data collection procedures**

After signing of the consent form, a structured questionnaire was then administered to all participants one at a time by the investigator before they were seen by the physician / gynecologist. The demographic data which includes the age, LMP, number of sexual partners e.t.c, clinical history and results of pelvic (cervix) examination was obtained, recorded and scored as shown in the Appendix (II & III).

### **3.10 Sample collection**

After the assurance, the patient was placed at a lithotomy position as shown in Appendix VI and a pelvic examination was conducted by the Nurse/Physician. A speculum was

lubricated with warm water and then inserted gently into the vagina and then it was slowly opened. This was done in order to have a clear view of the cervix. A Pap smear was then taken from the cervix (transformation zone) using a cervix broom as explained in Appendix V. The smear was prepared for routine work in the hospital laboratory in the usual manner where it was smeared on a frosted glass slide, fixed immediately using 95% alcohol, labeled and transported to the laboratory. Immediately after placing the routine smear on the slide, the cervix broom was dropped into a vial containing 10 -15 ml of Pap spin collection fluid.

### **3.11 Laboratory procedures**

In the laboratory the vial containing the specimen was vortexed in order for the sample to be released from the cervix broom and the cervix broom was then discarded. The liquid based method was used to prepare the smear.

The investigator and an assistant (laboratory technologist) prepared the specimens by splitting the specimen into two and transferring then into two micro funnels. The specimens were then identically labeled and spun using a Shandon cytospin 4. After centrifugation at 1500 revolutions per minute for 10 minutes, a thin layer of diagnostic cellular material collected on the glass slide. The smears were then fixed in 95% ethyl alcohol for 15 minutes before staining. The working principle of the Shandon cytospin 4 is as explained in Appendix VI.

Each split sample was given the same cytology number corresponding to the routinely stained smear. The same number for each slide appeared on the reporting form. The split samples were then separated and each slide put in different staining trays.

### **3.11.1 Staining**

One split smear was stained by the standard Pap staining procedure. The staining procedure consisted of 25 dishes, of which 8 contained 95% alcohol, 3 absolute alcohols and 2 contained alcohol-based counter-stains (OG 6 and EA). The other dishes contained water for rinsing among others as shown in Appendix VII.

The other smear was stained by the modified protocol (MP), where minimal alcohol was used (only for the initial fixation of smears and prior to mounting). In the rest parts of the procedure alcohol was replaced by 0.5 % acetic acid and also, one alcohol-based counter-stain Orange G was omitted from the procedure because it has been proved to add no diagnostic value of the stain and so its omission does not interfere with the results. <sup>(26)</sup> The haematoxylin used was preheated to 60°C to facilitate stain penetration and hence only a few staining dishes were used as shown in Appendix VIII.

After the staining the assistant who had already have been trained on the importance of coding and blinding then coded the slides for each staining method. The codes were only known by the assistant who revealed them at the end of data collection procedure. This was done for the purpose of blinding the readers to the staining method used for each case in order to reduce bias for any of the method during reporting.

### **3.11.2 Microscopy**

The two sets of trays were first examined by the investigator and a colleague for primary examination. The slides were then distributed to two cytopathologists / supervisors for the final screening and reporting. All was blinded as explained previously to the staining protocol. The reporting was done using The Bethesda System 2001 of reporting cervical smears as shown in Appendix IX. <sup>(27)</sup>

The results of the routine, standard and the modified Pap methods was compared and in case of any discrepancies, the smears were reviewed by two pathologists at the same sitting in order to come into a consensus before a final supplementary report was released to the requesting physician. The various nuclear and cytoplasmic parameters for staining quality and morphological details were assessed, scored and recorded as explained in the tables in Appendix III.

After attaining the sample size and the smear reporting is complete, the assistant then revealed the identity of the codes. The smears were then decoded by the investigator and each pair of smears were individually compared and the two protocols analyzed to assess the staining characteristics and cytodiagnosis using descriptive statistic and test of significance was determined.

### **3.12 Variables**

#### **3.12.1 Independent variables**

Modified Pap method

*Standard Pap method*

#### **3.12.2 Dependent variables**

Cytoplasmic staining characteristics

Nuclear and chromatin staining characteristics

Cervical smear results

### **3.13 Materials/Equipment**

#### **3.13.1 Equipment**

*Speculum, Cytospin, Microscope, Water bath or hot oven, staining rack, Coplin jars, slide trays, Computer and the accessories,*

#### **3.13.2 Supplies**

*Staining reagents (hematoxylin, Orange G, Eosin Azure), Alcohol, Scotts water, acetic acid, Xylene, DPX, cytofix, Writing materials (pencil, papers, pen), Cytobrush, Microscopic slides (frosted)*

#### **3.13.3 Personnel**

1. Two Pathologist/supervisors for re-examining the slides
2. A nurse and a gynecologist for Pap smear collection
3. Investigator and colleague for initial pap screening
4. One Statistician for data analysis
5. One Lab technologist as an assistant



### **3.14 Training procedures**

Training was done to familiarize the people involved in the screening with the new technique for better interpretation of the results. Also the assistant (lab technologist) was trained on coding system to be able to code the slides.

### **3.15 Quality assurance procedures**

All laboratory safety precautions were strictly adhered such as wearing of protective gears while working in the laboratory e.g. faces masks, grooves among others, washing of hands before leaving the laboratory, Not eating or drinking in the laboratory, Not spilling chemicals or specimen on the benches or floors, Not creating aerosols, Leaving the protective gear in the laboratory.

Standard Operating Procedures (SOP) was followed which included

Checking of reagents expiry dates before using them, proper preparation, labeling and storage of reagents, Proper maintenance of equipment like microscope, cytospin e.t.c., Following the right laboratory procedures as stated in the SOP.

### **3.16 Data collection instrument**

A structured questionnaire was used for data collection.

### 3.17 Ethical considerations

- Approval for authority to do the study was sought from Kenyatta National Hospital and University of Nairobi Ethics and Research Committee.
- Consent was sought from the participant for the investigator to administer the consent form and take the smear by giving full information about;
  - ✚ The purpose of the study.
  - ✚ The sample collection procedures, the benefits and risks of the study.
  - ✚ The duration of the study including the time the results may be available.
  - ✚ Other alternative methods other than the one used in the study.
- The participant were also be informed that;
  - ✚ Those who decline to participate will not be denied the necessary services offered within the hospital since it is a voluntary participation.
  - ✚ In case of a desire to withdraw from the participation there would be no penalty.
  - ✚ The participant would receive care in case of any injury from procedure
  - ✚ In case of any discrepancies, two pathologists reviewed the results in the same sitting and came to a consensus before the final results were released.
  - ✚ Those with abnormal smears were referred for colposcopy in the usual manner for further action and management.
  - ✚ The details of the study were confidential.
  - ✚ The details of the patient were kept in the soft copies and were password protected.

### **3.18 Data management and statistical analysis plan.**

The data was collected using a well structured questionnaire. It was pooled and entered into the excel file and exported into Statistical Package for Social Sciences (SPSS) version.

Analysis was mainly descriptive using both measures of central tendency (Mean, Mode, and Median) and measures of spread (ranges, variance, standard deviation) of various variables.

The coefficient of variation (CV) was used to measure relative spread of data obtained, to check the variability of results between the methods.

Results were then presented in graphs, charts, percentages and tables.

### **3.19 Study limitations**

#### **1. Inadequate funds to buy consumables.**

The study was self sponsored and some consumables were too expensive and unavailable in the country e.g cytofunnels for the cytospin. Acquiring them when they were finished proved to take too long and this almost grounded the project.

#### **2. Time limitation to evaluate the durability of the smears stained with the modified Pap method. Smears stained by any method are usually preserved for future references. The durability of these smears depends with the method of staining. For any new method, the durability needs to be assessed for a period of time to determine how long they will remain useful. This was not possible in this study due to limited time frame.**

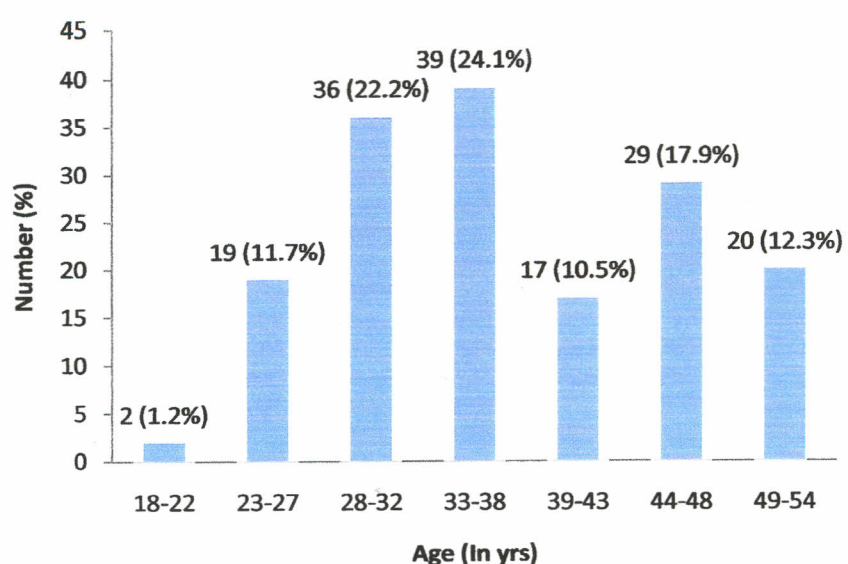
## CHAPTER FOUR: RESULTS

### 4.1 The socio-demographic data

Out of 162 paired smears examined in the study, 159 pairs were found satisfactory for evaluation. Three pairs of smears were excluded as they were unsatisfactory for evaluation due to scanty cellularity and a repeat was recommended.

The minimum age of the patients screened was 21 years and maximum was 49 years. The majority were between 33-38 years which was (24.1%). The mean age was 37.1, SD of 8.0 and a median of 36.5 (Figure 1).

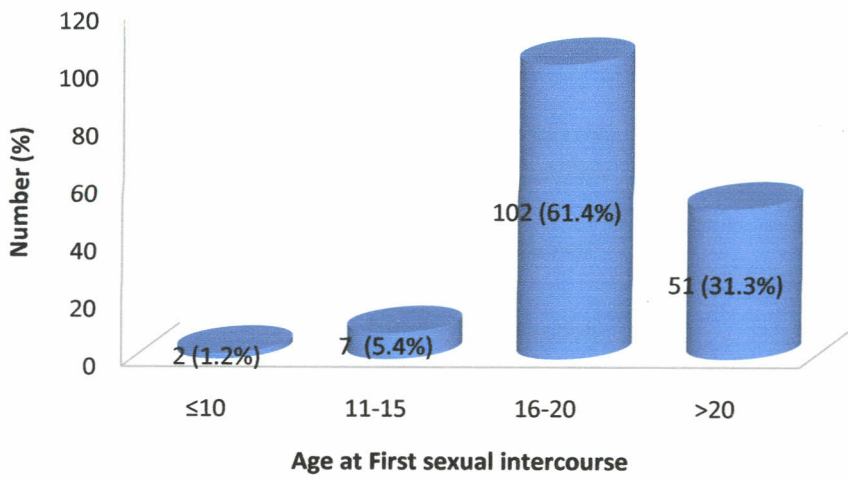
**Figure 1: Age distribution of the women screened for cervical cancer.**



### 4.3 Age at first sexual intercourse

The majority 102 (61.4%) had their first sexual intercourse at ages 16-20, 52 (31%) at ages more than 20 years and 2 (1.2%) had sexual intercourse at less than 10 years of age and below (Figure 3 and Table 1).

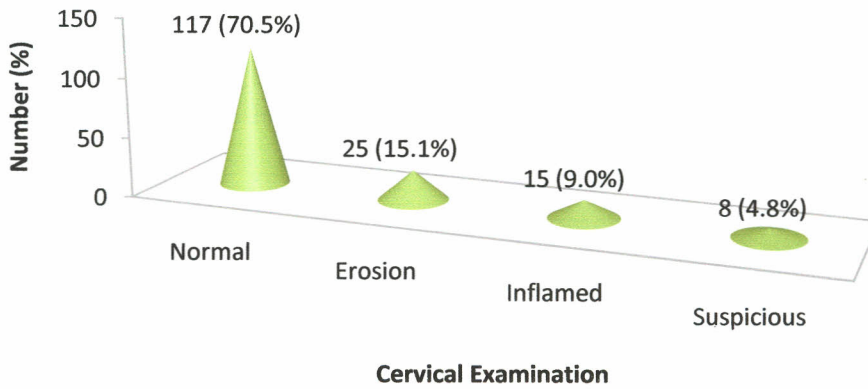
**Figure 3: Age at first sexual intercourse.**



#### 4.4 Cervical appearance on gross examination

On cervical examination, 117 (70.5%) were grossly normal, 8 (4.8%) were suspicious, 25 (15.1%) showed cervical erosion and 15 (9.5%) had inflammation (Figure 4).

**Figure 4: Cervical appearance on gross examination.**



#### **4.5 Risk factors for cervical cancer**

The risk factors for cervical cancer were also explored during the data collection and were summarized in table 1. Women who had had a previous cervical smear were 106 (65.4%), those with normal smears were 101 (91.4%), abnormal smears were 2 (3.6%) and 3 (5.35%) were unknown (never went back for the results).

The history of smoking was 1 (0.6%) and those with history of STI six months prior to the study were 9 (5.4%).

The use of contraceptive is currently 93 (54%) with IUCD used by the majority of women 61 (32.2%) followed by the oral contraceptive method (Table1).

**TABLE 1: Risk factors for cervical cancer of the study participants, n=162**

<b>Characteristic (variables)</b>	<b>Numbers (n)</b>	<b>Percent (%)</b>
1.Previous cervical smear		
No	106	65.4
Yes	56	34.5
2.Normal or Abnormal Previous Pap test		
Normal	51	91.4
Abnormal	2	3.6
unknown	3	5.35
3. History of previous STI (Last 6 months)		
No	153	94.6
Yes	9	5.4
4.History of Smoking		
No	161	99.4
Yes	1	0.6
5.Use of contraceptives		
No	69	35.5
Yes	93	58.4
5.1Contraceptives		
None	30	18.5
Natural	9	5.4
OCP	33	19.9
Condom	10	6.0
IUCD	61	32.3
Injectables	6	3.6
BTL	6	3.6
Jadelle	7	4.2
6. Age at First sexual intercourse		
≤10	2	1.2
11-15	9	5.4
16-20	102	61.4
>20	52	31.3
7. Number of sexual Partners (Last 6 months)		
None	19	11.4
One	136	84.6
none	6	3.7



#### **4.6 Quantified cost and time for the two methods**

The time taken to stain a bunch of Pap smear (25 slides) in the modified Pap method was  $3 \pm 0.5$  minutes while in the standard Pap method the time taken was  $20 \pm 0.5$  minutes.

This showed that the MP has a better turnaround time than the SP.

Ideally, for a complete set of standard Pap stain regardless of number of smears, there are eleven containers of alcohol and each container holds approximately half a liter of alcohol. This means 5.5 liters of alcohol are used per one Pap procedure. For better staining quality the reagents should be changed periodically due to transfer of stains from one container to another. This is particularly so for alcohol which may become cloudy or colored (contamination by water or stains) interfering with the staining quality. The periodicity of change is dependent on the volume of smears stained by each batch of reagent. In this study the change was done once after two weeks and this justifies the use of ten liters of alcohol in the standard Pap. The 95% alcohol for fixation was not changed since the smears were already fixed when they came to the laboratory and therefore the slides took only a few minutes in the alcohol instead of the usual 15 minutes .

In the modified Pap procedure only the 95% alcohol before the mounting was changed and therefore only 1.5 liters were used i.e one liter before mounting for the two changes and 0.5 liters of 95% alcohol before staining. The cost for one liter of alcohol was Ksh 2000 and therefore ten liters costs Ksh 20000 while one and half liters cost Ksh 3000.

In MP procedure 0.5% acetic acid was used instead of alcohol. For the two changes of reagents only 10 milliliters of acetic acid was used. One liter of acetic acid cost Ksh 1500 and therefore the cost of 10 milliliters of acetic acid is Ksh 15.

Therefore in MP pap the cost of alcohol per smear was Ksh 18.50 and in the SP the cost was 123.45 per smear (for 162 smears). If the cost of both alcohol and acetic acid is combined for the MP it will amount to Ksh  $(.90 + 18.50) = \text{Ksh } 19.40$  (Table 2).

**Table 2: Quantified cost and time for the two methods**

	<b>Modified Pap</b>	<b>Standard Pap</b>
<b>Time (minutes)</b>	3 ± 0.5	20 ± 0.5
<b>Reagents &amp; amount</b>	1.5 liters of alcohol 15 mls of acetic acid	10 liters of Alcohol
<b>Total cost of the alcohol used</b>	Ksh 3000	Ksh 20000
<b>Total cost of the acetic acid used</b>	Ksh 15	0
<b>Cost/Pap smear for alcohol</b>	KSH 18.50	KSH 123.45
<b>Cost/pap smear for Acetic acid</b>	90 cents	
<b>Cumulative cost per slide</b>	Ksh 19.40	Ksh 123.45

#### 4.7 Cytomorphologic features.

The staining quality was compared in both methods and the P-value in the staining of the cytoplasmic borders and nuclear borders shows no statistically significant difference in both methods. In the staining of the cytoplasm and nuclear chromatin the P-value indicated that there was statistically significant difference in both methods (Table 3).

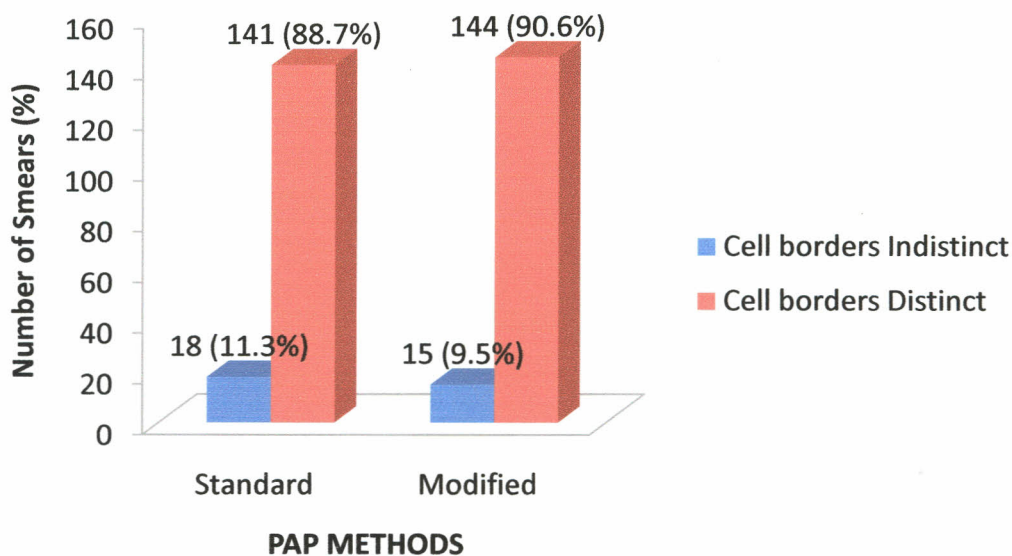
**Table 3: Cytomorphologic features**

PARAMETER	STANDARD PAP	%	MODIFIED PAP	%	P-Value
1.Cell/Cytoplasmic borders					
Indistinct	18	11.3	15	9.4	$\chi^2(0.30), P=0.71$
Distinct	141	88.7	144	90.6	
2.Cytoplasmic Staining					
Unsatisfactory	14	8.8	10	6.3	$\chi^2(36.4), P=0.00 <0.05$
Satisfactory	102	64.2	53	33.3	
Excellent	43	27.0	96	60.4	
3.Nuclear borders					
Indistinct	7	4.4	5	3.1	$\chi^2(0.35), P=0.77$
Distinct	152	95.6	154	96.9	
4.Chromatin staining					
Hazy	79	49.7	54	34.0	$\chi^2(8.08), P=0.006 <0.05$
Distinct	80	50.3	105	66.0	

#### 4.8 Staining of the Cell borders

18 (11.3%) of the smears had indistinct cell borders and 141 (87%) had distinct cell borders in the standard pap method. In modified Pap method 15 (9.5%) of the smears had indistinct cell borders and 144 (90.6%) had distinct cell borders (Figure 5).

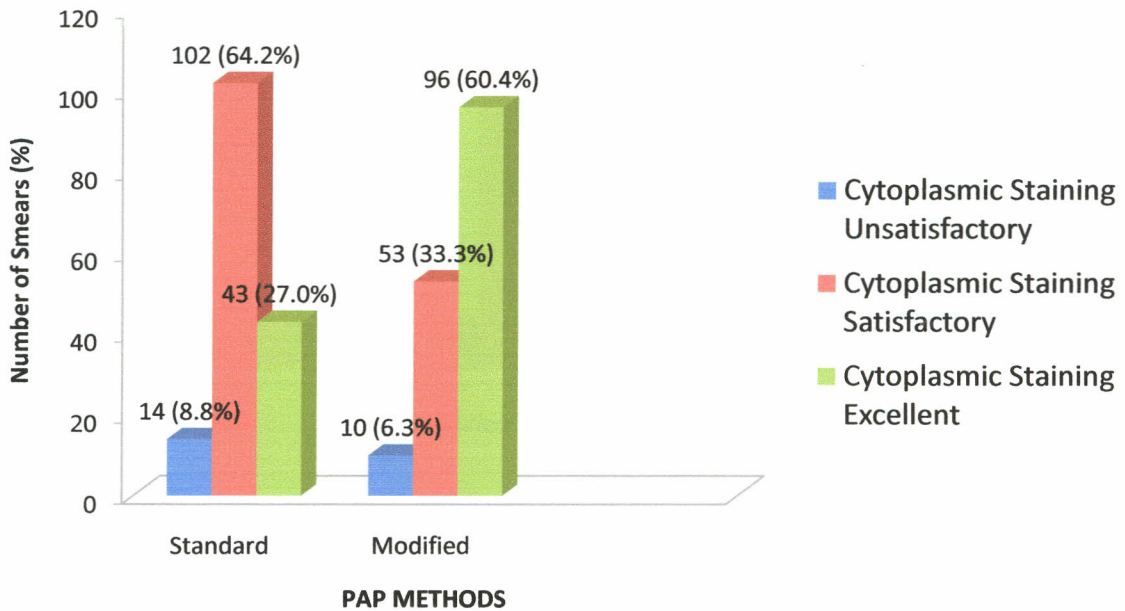
**Figure 5: Staining of the Cell borders**



## 4.9 Cytoplasmic Staining

The standard Pap method had 14 (8.8%) unsatisfactory cytoplasmic staining, 102 (64.2%) satisfactory and 43 (27.0%) were excellent. Modified Pap method had 10 (6.3%) in the unsatisfactory cytoplasmic staining, 53 (33.3%) satisfactory and 96 (60.4%) stained excellently (Figure 6).

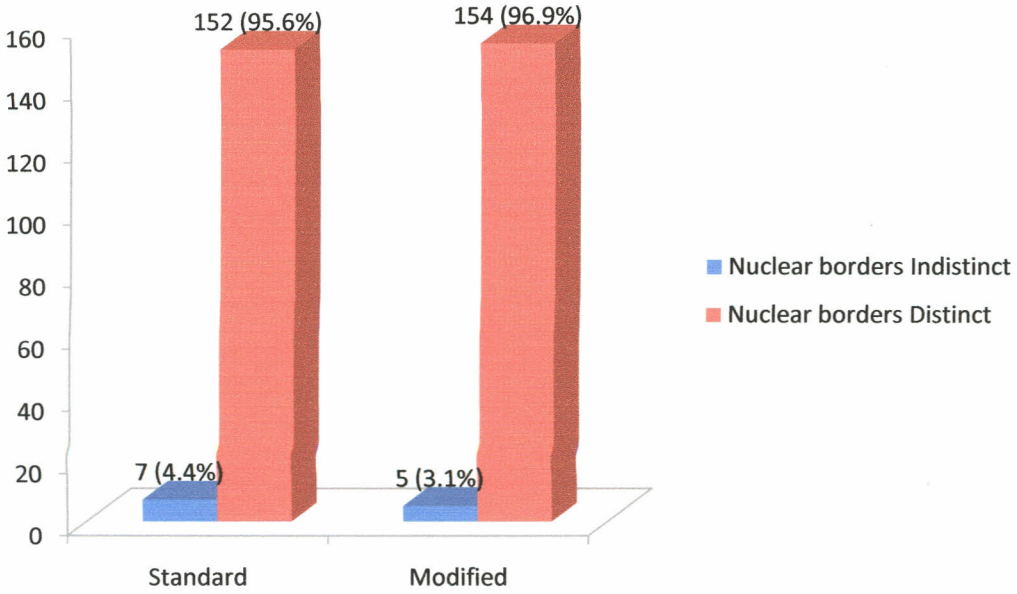
**Figure 6: Cytoplasmic Staining**



#### 4.10 Staining of nuclear borders

The nuclear membranes/borders were well stained in both methods with just a slight variation. In SP 95.6% nuclear borders were distinct and 4.4% were indistinct. 96.9% were distinct and 3.1% indistinct in the MP (Figure 7).

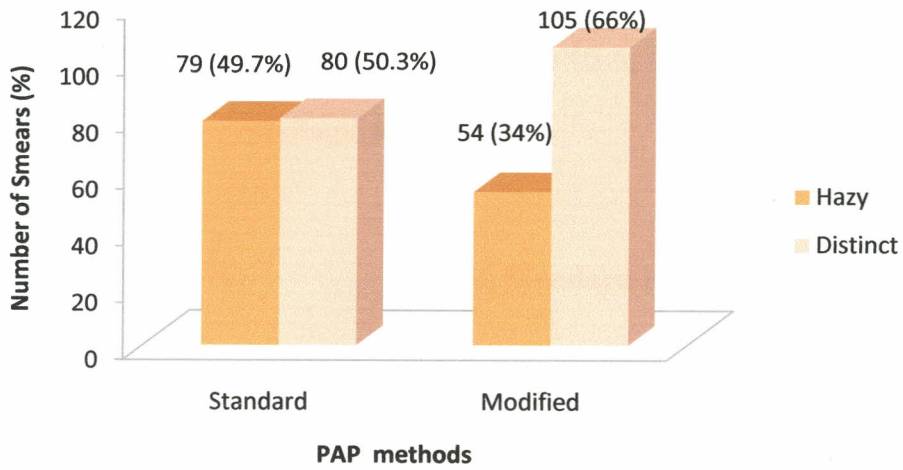
**Figure 7: Staining of nuclear borders**



#### 4.11 Chromatin staining (appearance)

In the standard Pap the nuclear staining was hazy in 79 (49.7%) and distinct in 80 (50.33%), while in modified Pap nuclear staining was hazy in 54 (34%) and distinct in 105 (66%) in SP (Figure 8).

**Figure 8: Chromatin staining (appearance)**



#### **4.12 Photomicrograph: cytomorphologic features**

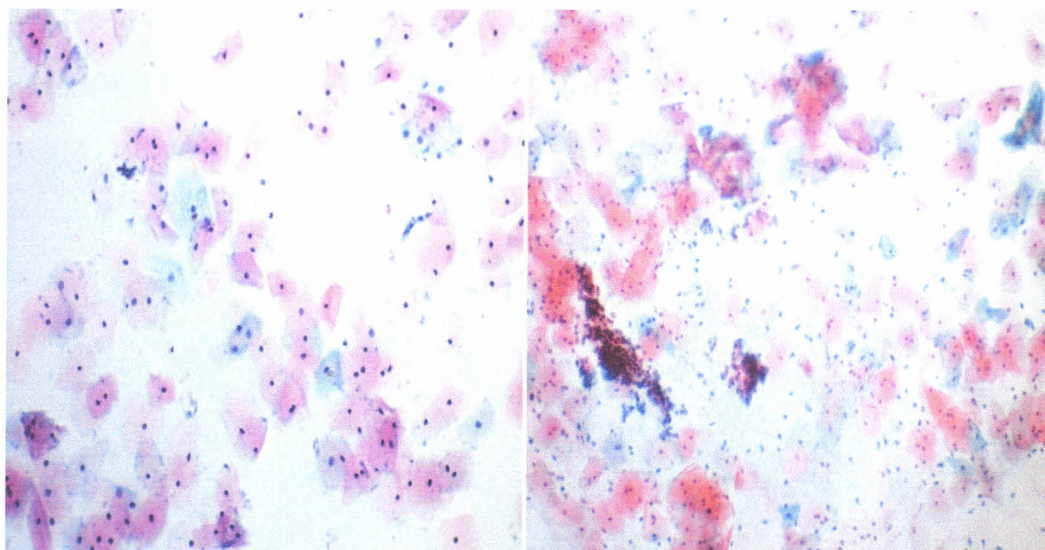
The simplified presentation with two cytoplasmic colours (blue / green and pink) in the modified Pap instead of the usual three seen in standard Pap was quite acceptable to the technologists, cytologist and pathologists. The pink colour of keratin seen in the modified Pap is similar to that seen with haematoxylin and eosin stain, and therefore the cytologists/pathologists were comfortable in reading the smears. Organisms (e.g candida, Bacterial vaginosis) and the epithelial cell abnormalities could be interpreted by pathologists without any diagnostic difficulty (Figure 10 A, B & C).

#### **Photomicrograph: cytomorphologic features**

##### **1. Normal Pap smear**

**(i) Modified Pap ( $\times 10$ )**

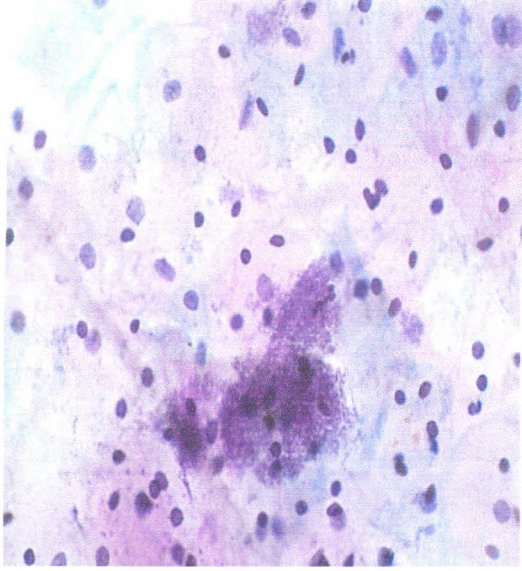
**(ii) Standard Pap ( $\times 10$ )**



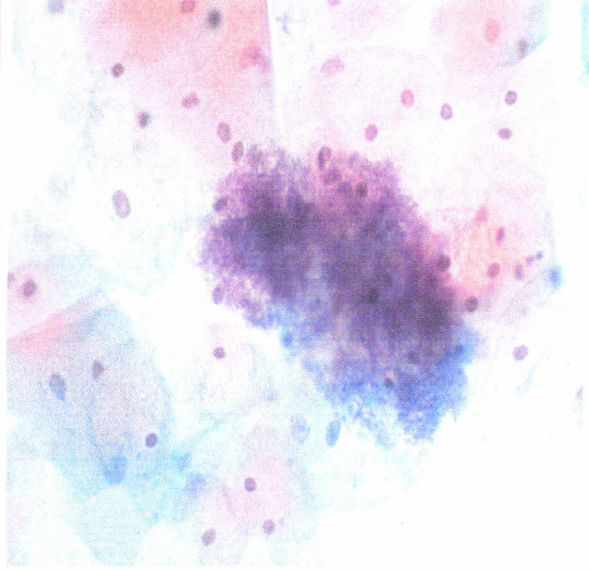


## 2. Clue cells

(i) Modified Pap ( $\times 25$ )

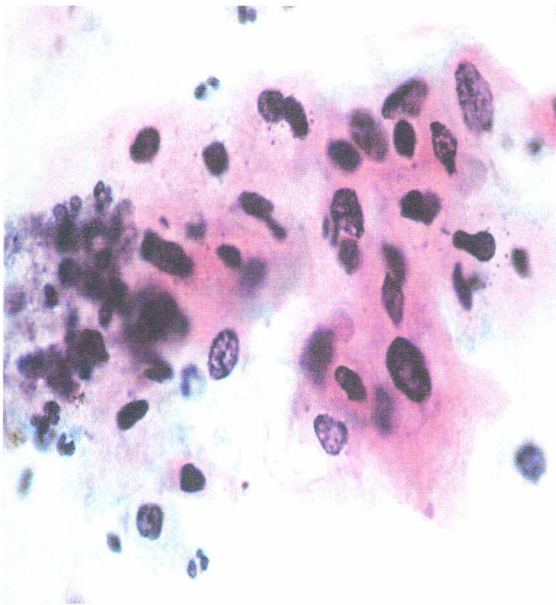


(ii) Standard Pap ( $\times 25$ )

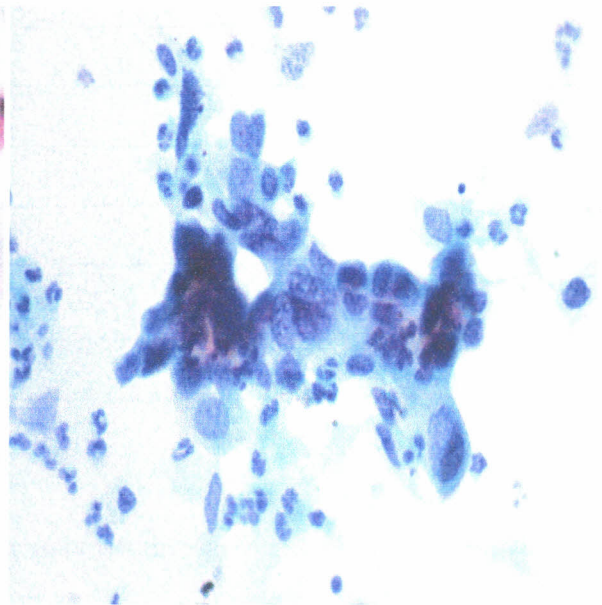


## 3. High grade squamous intraepithelial lesion

(i) Modified Pap ( $\times 40$ )



(ii) Standard Pap ( $\times 40$ )



### 4.13 Pap smear results (cytodiagnosis)

In this study the diagnoses concurred in all cases in the paired smears and the pathologists/cytologists encountered no difficulties in the interpretation of the smears stained by the modified Pap.

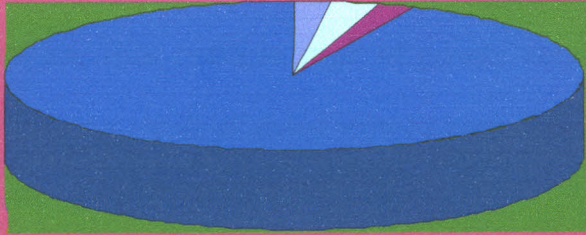
There were 152 (95.6%) negative smears for intraepithelial lesion or malignancy, and 7 (4.4%) epithelial cell abnormalities (ASCUS, LSIL and HSIL). 1.25%, 1.89% and 1.25% were ASCUS, LSIL and HSIL respectively. Inflammatory and reactive changes were 96 (60.37%) (Table 3 and Figures9).

**Table 3: Pap smear results (Cytodiagnosis)**

CYTODIAGNOSIS	NUMBER OF CASES	PERCENTAGE (%)
NILM	152	95.6%
ASCUS	2	1.26
ASCUS-H	0	0
AGC-NOS	0	0
LSIL	3	1.89
HSIL	2	1.26
SCC	0	0
TOTAL	159	100
Others (inflammation & reactive)	96	60.37

**NB:** The inflammation and reactive change may occur together with the lesions and that is why the totals do not add up.

**Figure 9: Pap smear results (Cytodiagnosis)**



■ ASCUS	- 1.25%
■ ASCUS-H	- 0
□ AGC-NOS	- 0
■ LSIL	- 1.89%
■ HSIL	- 1.25%
■ SCC	- 0
■ NILM	- 95.6%

## **CHAPTER FIVE: DISCUSSION, CONCLUSION AND RECOMMEDATIONS**

### **5.1 DISCUSSION**

The Pap smear has been utilized for cervical cancer screening for more than 50 years. Pap smear programs are complex and costly to run and have failed to reach a significant proportion of women. <sup>(13)</sup> The conventionally practiced method (standard Pap) is complex, with multiple steps of greatly varying times, making it liable to laboratory errors. As a result of above reasons papanicoloau staining method has been modified in several ways. <sup>(4)</sup>

This study was to compare the modified Pap and the standard Pap methods in the screening of the cervical smears. The modified Pap sought to improvise on the conventionally practiced Pap staining technique (standard Pap) by replacing the alcohol with 0.5 % acetic acid in all the steps except the initial fixation and prior to mounting the smears.

The participants in the study were 162 of whom 159 smears were satisfactory for evaluation and three were unsatisfactory where a repeat was recommended.

The ages of the women who participated in the study ranged between 22-49 years, majority being in the ages 33-38 years. The mean age of the participant was 37.1, standard deviation of 8.0 and a median of 36.5 years. On Pap screening only 34.5% of the women had ever had Pap test and 91.4% of these Pap smears were normal. This is slightly lower than in a study done by Gatune et al., <sup>(28)</sup> at Tigoni, Kenya who found out

that 67% of those screened had had a pap smear. The mean age of those who were screened was the same that is 37.1.

The study showed that the time taken to stain each bunch Pap smear slides (25 slides, 40 slides depending on the slide carrier) in the modified Pap method was reduced considerably to  $3 \pm 0.5$  minutes while in the standard Pap method the time taken was  $20 \pm 0.5$  minutes for the same number of slides. This concurred with a study by Gupta et al.,<sup>(22)</sup> for both SP and MP staining time.

Ideally, for a complete set of standard Pap stain, there are eleven containers of alcohol regardless of number of smears and each container holds approximately half a liter of alcohol. This means 5.5 liters of alcohol are used per one Pap procedure. For better staining quality the reagents should be changed periodically due to transfer of stains from one container to another. This is particularly so for alcohol which may become cloudy or colored (contamination by water or stains) interfering with the staining quality. The periodicity of change is dependent on the volume of smears stained by each batch of reagents. In this study the change was done once after two weeks and this justifies the use of ten liters of alcohol in the standard Pap. The 95% alcohol for fixation was not changed since the smears were already fixed when they came to the laboratory and therefore the slides took only a few minutes in the alcohol instead of the usual 15 minutes.

In the modified Pap procedure only the 95% alcohol before the mounting of slides was changed and therefore only 1.5 liters were used i.e one liter before mounting for the two

changes and 0.5 liters of 95% alcohol before staining. The cost for one liter of alcohol was Ksh 2000 and therefore ten liters costs Ksh 20000 while one and half liters cost Ksh 3000.

In the MP procedure, 0.5% acetic acid was used instead of alcohol. For the two changes of the reagents only 10 milliliters of acetic acid was used. One liter of acetic acid cost Ksh 1500 and therefore the cost of 10 milliliters of acetic acid is Ksh 15.

Therefore in MP pap the cost of alcohol per smear was Ksh 18.50 and in the SP the cost was 123.45 per smear for (162 smears). If the cost of both alcohol used and acetic acid (which replaced the alcohol) is combined in the MP it will amount to Ksh  $(.90 + 18.50) =$  Ksh 19.40 per smear. This shows a drastic reduction of 1/7 of the total cost of staining in the modified pap compared to standard Pap method.

This is similar to a study done by Gupta et al <sup>(22)</sup> which used 1% acetic acid to replace alcohol which also showed that it took 3-4 minutes to stain using the modified Pap. Thus a greater number of slides can be stained each day without additional input. The cost/smear was INR 70 (£0.89) which is equivalent to Ksh 71.2 in the SP and INR 12 (£0.15) which is equivalent to Ksh 12 in the MP. This shows a reduction to 1/6 of the total cost in MP compared to SP method.

The acetic acid used in the MP acts as a mild dehydrating agent which is the same role played by the alcohol in the standard Pap method. Dehydration prepares the smear for the staining by removing the excess water. The main advantages of using acetic acid in place of alcohol are its easy availability and extremely low cost as shown in the study. The

simplicity of the procedure (uniform 10 dips at each step) also reduced the risk of errors while staining because there is no variation of time or dips from one staining container to the other unlike in the standard Pap where each container has different timings during staining.

The staining quality of both nuclear chromatin and cytoplasm in our modified protocol was found to be better than the standard Pap protocol where the P-value in both cases was less than 0.05. This result disagrees with a study done by Gupta et al <sup>(22)</sup> which showed a P value of 0.69 for cytoplasmic staining and a P value of 0.55 for chromatin staining indicating that there is no statistical significance difference in both cytoplasmic and chromatin staining. The results however concur with a similar study done in India by Roy et al.<sup>(16)</sup> In this study referred to as rapid economic acetic acid Pap (REAP) technique 1% acetic acid was used to replace acetic alcohol instead of 0.5%. In the REAP protocol 110 pairs of smears were used to compare the two methods. The cytoplasmic and nuclear staining was optimal in 100 and 105 smears respectively while 90 and 100 were optimal in the standard pap respectively thus making the modified pap better than the standard Pap method. The results for the cytodiagnosis concurred in both methods.

This was similar to studies by Gupta et al., <sup>(22)</sup> and Roy et al., <sup>(16)</sup>. In both modifications the Pap smear results were the same with the results of the standard Pap method.

The results showed that 152 (95.6%) were negative for intraepithelial lesion or malignancy and 7 (4.4%) had epithelial cell abnormalities (ASCUS-1.26, LSIL-1.89, and HSIL-1.26). The highest number of abnormal Pap smears was in the age group 38-49

years. These concurred in a study done by Waweru et al.,<sup>(29)</sup> which showed ASCUS to be 3.7% and HSIL to be 1.5% with the slight variation being probably due to the small sample size in this study.

Other few shorter staining methods such as Rapid Pap<sup>(19)</sup> and Ultrafast Pap<sup>(20)</sup>, have been tried successfully in the past to reduce the staining time and showed similarities in both methods in terms of cytomorphology and cytodiagnosis. However, these procedures use large volumes of alcohol, which is costly and difficult to procure in many developing nations.



## **5.2 Conclusion**

1. Modified Pap method uses few reagents which are less costly, takes a shorter time to stain and therefore it is economical in poor resource settings.
2. The modified Pap method meets the criteria of a good cytological stain by showing better cytoplasmic staining and sharp nuclear chromatin staining than the standard Pap method.
3. Modified Pap method showed a diagnostic accuracy similar to the standard Pap method and therefore it is a suitable method for the screening of cervical smears.

### **5.3 Recommendations**

From the study the following recommendations may be made;

1. Modified Pap method should be adopted in our laboratories since it is inexpensive and less time consuming in the screening of cervical cancer.
2. To assess the smears stained with modified Pap method for some duration of time to determine their durability for future reference incase of any result dispute.

## REFERENCES

1. Ralph M. Richart. Screening. The next century cancer. Supplement, cancer 1995; Volume 76, 1919-1927
2. Be'atrix C, Catherine L, Patricia C et al. Cost-effectiveness of monolayers and human papillomavirus testing compared to that of conventional Papanicolaou smears for cervical cancer screening: *Diag. Cytopathology*, 2001; 24: 412-420.
3. Asthana S and Satyanarayana L. Prevention and control of uterine cervical cancer, current strategies. *Indian J. Community Med*, Nov 30 2007; 32:233-4
4. Leopold G. Koss. Diagnostic cytology and its histopathologic bases. Volume two: 1474-1475 Fourth edition. J.B. Lippincott Company Philadelphia.
5. Renshaw A, Sack M, Sharamitaro N et al. Cervical cytology practice guideline. American Society of Cytopathology, *Diag. cytopathology*, 25: 3-24.
6. Girianelli V R and Thuler L C S. Evaluation of agreement between conventional and liquid-based cytology in cervical cancer early detection .Brazilian National Cancer Institute. *Diag. Cytopathol.*2007; 35:545–549.
7. Gichangi P, Estambale B, Bwayo J et al Knowledge and practice about cervical cancer and Pap smear testing among patients at Kenyatta National Hospital, Nairobi, Kenya. *Int. J Gynecol Cancer* 2005; 15: 1049–1059.
8. Malihe H and nadereh B. Cervical cancers screening. Tehran 14194, Iran. April 2005. [valrec2@yahoo.com](mailto:valrec2@yahoo.com). 2/2/2010.
9. Hamont VD, Beckkers RLW, Massuger LFAG et al. Detection, management and follow-up of pre-malignant cervical lesions and the role of human papillomavirus. *J. Med. Virol*, 2005; 76:470-475.
10. Nguyen HN and Staffan RB, Nordqvist. The Bethesda system and evaluation of abnormal Pap smears oncology associates, Sheridan Health Care Corporation, Hollywood, Florida Gynecologic Seminars in Surgical Oncology 1999; 16:217–221.
11. Rika Y, Toshiyuki S, Kirumbi WL et al. Human papillomavirus infection and cervical abnormalities. *J. Med. Virol*, 2008; 80:847-855

12. Smita NJ, Gopalkrishna V, Kishore KB, et al. Screening cervical squamous intra-epithelial changes and human papillomavirus infection in women infected with human immunodeficiency virus in Pune, India. *J. of Med. Virol*, 2005; 76:470–475.
13. Sylvia T, Louise K, William D et al. Direct comparison of liquid-based and conventional cytology in a South African screening trial. *Int. J. Cancer*, 2006; 118, 957–962.
14. Sarian L O, Derchain S F, Naud P. Evaluation of visual inspection with acetic acid (VIA), Lugol's iodine (VILI), cervical cytology and HPV testing as cervical screening tools. *J Med Screen* 2005; 12:142–149.
15. Buncha P. Alternative cervical cancer prevention in low-resource settings: Experience of visual inspection by acetic acid with single visit approach in the first five provinces of Thailand: Australian and New Zealand. *J. Obstetrics and Gynecology*, 2007; 47:54-60.
16. RoyBiswas R, Chandi CP, Ramprasad D et al. Rapid economic, acetic Acid, Papanicolaou Stain (REAP) - Is it suitable alternative to standard Pap stain? *J. Med. Sci*, 2008; Volume 1, No.2.
17. Gill GW. Enviro-Pap: an environmentally friendly, economical, and effective Pap stain. *Lab Med Indianapolis*, 2006; 37:105–8.
18. Pandit AA and Pravin BS. Application of modified ultrafast Papanicolaou stain in cytology of various organs. *Diagnostic Cytopathology*, Vol 34, No 2.
19. Sato M, Taniguchi E, Kagiya T et al. A modified rapid papanicolaou stain for imprint smear. *Acta Cytologica*, 2004; 48:461-462.
20. Yang GCH and Alvarez II. Ultrafast Papanicolaou Stain--An alternative preparation for fine needle aspiration. *ACTA Cytol*, January-February 1995; vol. 39, no. 1.
21. Yang GCH et al. Ultrafast Papanicolaou protocol for cervicovaginal smears. *ASC Abstracts*, Nov. 1, 1995.
22. Gupta S, Chachra KL, Bhadola P et al. Modified Papanicolaou staining protocol with minimum alcohol use: a cost-cutting measure for resource limited. *Division*

## **APPENDICES**

### **APPENDIX I: CONSENT FORM**

#### **TITLE OF STUDY**

#### **A COMPARISON OF MODIFIED AND STANDARD PAP STAINING METHODS IN THE ASSESSMENT OF CERVICAL SMEARS**

##### **Consent explanation for patients aged 18 – 49 years**

I am Rose Gachie a Master's student in Clinical Cytology at the University of Nairobi. I am conducting a study about screening of the cervical cancer. This procedure will help you to know whether you may be having cervical neoplasia or not and future follow ups. A specimen will be taken from your cervix by the doctor/nurse during a speculum examination of your cervix using a sampler known as cervix broom which is sterile and safe. The specimen will be analyzed in Cytology laboratory in the usual manner for your pap smear and the rest of the sample will be used in a study to compare screening techniques at the University of Nairobi laboratory. This will help us to identify a method which may be cheaper and may take a shorter time to complete. The slides will be stored at cytology laboratory up to five years for further evaluation if deemed necessary with permission from KNH/UON/ERC. This study has been approved by KNH/UON/ERC.

##### **Risk / benefits**

During this procedure there may be a slight discomfort. There are no monetary benefits and neither will you incur any extra cost. The results of the study will be communicated to the cytology laboratory department/pathologist conducting the routine Pap smear. This is for the purpose of comparing the results and coming into a consensus before the final results are released. The study will benefit you in that you will receive an additional Pap

1 smear result which will show whether you are at risk of developing cervical cancer. The results will be sent to your file in the clinic. The results of the whole study will also be disseminated to Ministry of Public Health for possible change. These results will benefit the nation if the method proves to be cheaper for screening of cervical cancer.

### **Management of the results**

In case of any abnormal smear, you will be referred to the colposcopy clinic in the usual manner for further management. All abnormal result will be flagged for action to be taken.

### **Participant's rights**

Your participation in this study is voluntary and if you decline to participate, you will not be denied any services that are normally available to you in this hospital. In case you wish to withdraw from participation there will be no penalty.

### **Duration of participation**

Only one specimen will be taken from you for this study, you will return to collect your results on your next clinic appointment.

### **Assurance of confidentiality of volunteer's identity**

Records relating to your participation in the study and results will remain confidential.

You will be given a consent form to sign and you may keep a copy for your own results.

**Patient’s declaration**

I -----hereby agree to participate in the study being carried out by Mrs. Rose. N. Gachie. I agree a sample to be taken for examination as explained to me. I understand that i will not suffer any extra discomfort over and above what is required for the usual screening and i will not pay any extra cost for the Pap smear. Any results that would help in the management of my condition will be communicated to me. I also understand that I may withdraw from the study at anytime and my withdrawal will not in any way deny me any health benefits to which I am entitled.

Signed -----

Or thumb print-----

Date: -----

Signature of principal investigator (or authorized representative) .....

**Contact information**

If you have questions now or in the future regarding your rights or this study or research related injury, you may ask any of the field officers involved in this study or contact the investigator Rose Gachie MSc student at the University of Nairobi on 0722438368 or Chairperson, KNH/UON/ERC. P.O BO, 20723-00200 Nairobi. Tel#:020-2725272.

[A copy of this form to be given to the client to keep]

## APPENDIX II: QUESTIONNAIRE

Date.....

### STUDY TITTLE

A COMPARISON OF MODIFIED AND STANDARD PAP STAINING METHODS IN  
THE ASSESSMENT OF CERVICAL SMEARS

1.1 Eligibility criteria (exclusion if yes to);

1.2 History of total hysterectomy

No  0 Yes  1

To continue only if eligible

---

2. Study number: .....

3 Laboratory number: .....

4. Last monthly period (date): .....

5. Age (years).....

6 Postmenopausal:

No  0 Yes  1

7. History of hormonal replacement therapy:

No  0 Yes  1

8. Age at first sexual intercourse

$\leq 10$   0      11-15  1      16-20  2       $>20$   3

9. Number of sexual partners (last 6 months)

None  0      One  1       $> One$   2



10. History of previous STI (Last 6 months)

No  Yes

11. History of smoking

No  Yes

12. 1 Previous Pap test:

No  Yes

12.2 If yes, Normal  Abnormal

13. 1 Use of contraceptives No  Yes

32.2 If yes specify Natural  OCP

Condom  IUCD  Injectables  Others

14. 1 History of previous malignancy No  Yes

14.2 If yes, specify.....

15. History previous radiotherapy (last 6 months) No  Yes

16. Cervix examination: Normal  Erosion  Inflamed  suspicious

**APPENDIX III: RESULTS FORM**

INTERPRETATION OF THE RESULTS FOR ROUTINE, STANDARD AND  
MODIFIED PAP STAINING METHODS

STUDY NO. -----

Papanicolaou smear

Specimen adequacy (method F)

Cytoplasmic staining: Unsatisfactory	<input type="checkbox"/>	Satisfactory	<input type="checkbox"/>	Excellent	<input type="checkbox"/>
Nuclear borders: Indistinct	<input type="checkbox"/>	Distinct	<input type="checkbox"/>		
Chromatin Hazy	<input type="checkbox"/>	Distinct	<input type="checkbox"/>		

Interpretation of the results

Comment -----

Specimen adequacy (Method R)

Cytoplasmic staining: Unsatisfactory	<input type="checkbox"/>	Satisfactory	<input type="checkbox"/>	Excellent	<input type="checkbox"/>
Nuclear borders: Indistinct	<input type="checkbox"/>	Distinct	<input type="checkbox"/>		
Chromatin Hazy	<input type="checkbox"/>	Distinct	<input type="checkbox"/>		

Interpretation of the results -----

Comments-----

Investigators sig: -----supervisors Sig: -----date-----

Consensus report (QC) -----

**APPENDIX IV: DUMMY TABLES OF THE RESULTS**

**TABLE (1): RESULTS PER SMEAR**

	NORMAL	ASCUS	ASCUC-H	LSIL	HSIL	SCC	AGUS-NOS	OTHERS(specify)
<u>METHODS</u>								
ROUTINE PAP								
SECRET CODE								
SECRET CODE								

**KEY**

- NILM - Negative for intraepithelial lesion or malignancy
- ASC-US - Atypical squamous cells of undetermined significance
- ASC-H - Atypical squamous cells—cannot exclude high-grade lesion
- AGC-NOS - Atypical glandular cells, not otherwise specified
- LSIL - Low-grade squamous intraepithelial lesion
- HSIL - High-grade squamous intraepithelial lesion
- SCC - Squamous cell carcinoma

Any discrepancy between the results is addressed as previously explained.

**TABLE (2): CYTOMORPHORLOGICAL FEATURES**

PARAMETER	SCORES	CONVENTIONAL PAP	%	MODIFIED PAP	%	P VALUE
1.Cell borders						
Indistinct	1					
Distinct	2					
2.Cytoplasmic staining						
Unsatisfactory	0					
Satisfactory	1					
Excellent	2					
3.Nuclear borders						
Indistinct	1					
Distinct	2					
4. Chromatin						
Hazy	1					
Crisp	2					

**TABLE (3): DUMMY TABLE OF CYTODIAGNOSIS**

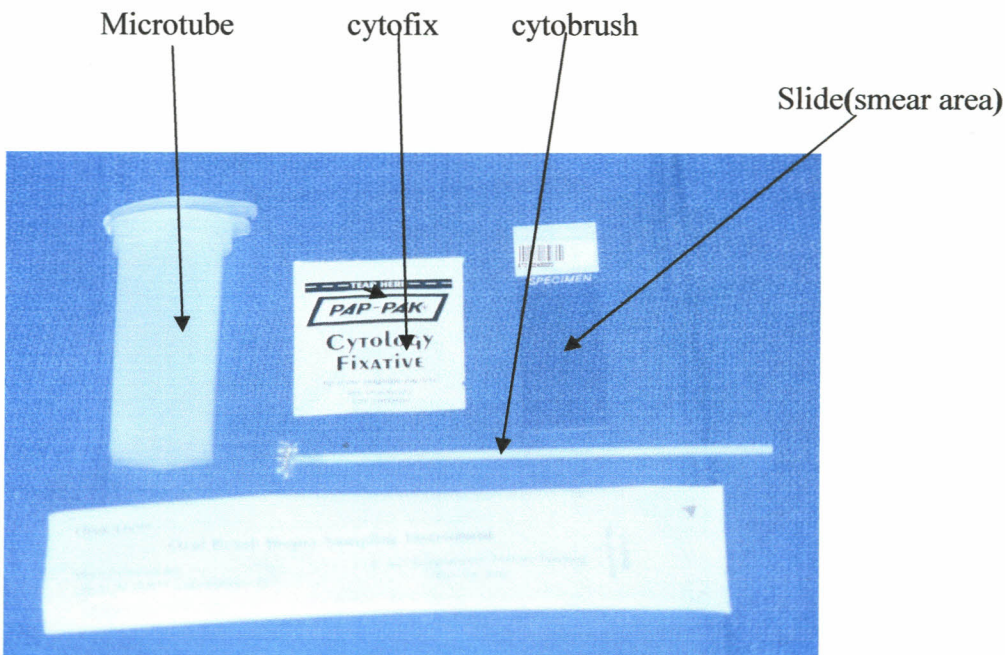
SPECIMEN ADEQUACY	SCORES	STANDARD PAP	%	MODIFIED PAP	%	TOTAL
UNSATISFACTORY SMEAR	0					
SATISFACTORY SMEAR	2					
<u>CYTODIAGNOSIS</u>						
NSIL	1					
ASCUS	2					
ASCUS-H	3					
AGC-NOS	4					
LSIL	5					
HSIL	6					
SCC	7					

The specimen adequacy for scoring will be determined by  $\geq 8$  intermediate cells per field.

## APPENDIX V: WORKING PRINCIPLE OF A SHANDON CYTOSPIN 4

Using centrifugal principle, the Cytospin deposits cells onto a clearly-defined area of a glass slide and allows for the absorption of the residual fluid into the sample chamber's filter card. Cyto centrifugation also constructively flattens cells for excellent nuclear presentation. During operation, the instrument's spinning action tilts Cytofunnels upright and deposits a thin layer of cells on the slide, giving all cell types equal opportunity for presentation. In the Load or Stop position, this unique tilting feature reduces cell loss by preventing residual fluid from coming into contact with the prepared slide. It uses proven, low-speed centrifugation to concentrate and deposit a thin layer of cells onto a microscope slide to ensure better cell capture and better preservation of cell morphology.

### The liquid based sample preparation and apparatus

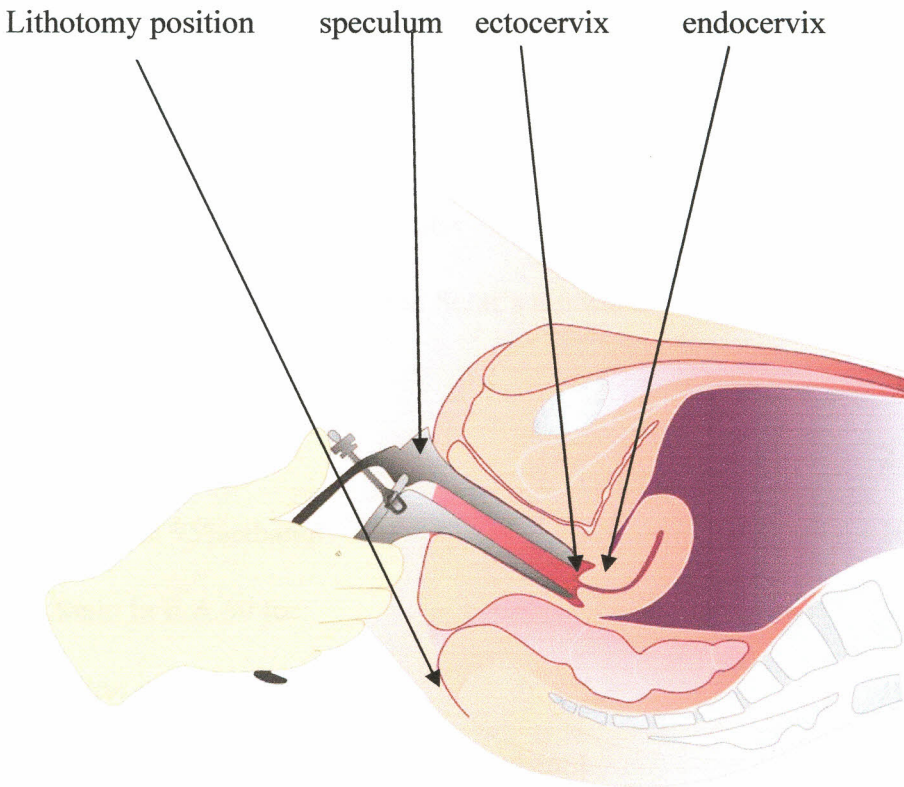


Source: Spears/Gillette 2010

## APPENDIX VI: PAP SMEAR COLLECTING PROCEDURE

Upon signing of the consent form and patients assurance, the patient is placed on examination table in a lithotomic position. The speculum is then wet with water and is inserted into the vagina until the cervix comes into full view. The cervix broom is inserted and is rotated at 360 degrees at the squamous columnar junction or any suspicious area. It's then removed and a smear is made on a slide or is placed in the cytofix for further processing. The speculum is slowly withdrawn and removed; the patient is then advised to come for the results during the next visit to the clinic.

### Pap smear collection procedure



Source: Spears/Gillette 2010

## APPENDIX VII: STANDARD PAPANICOLOAU STAINING METHOD <sup>(4)</sup>

### Principle of the stain

Haematoxylin stains the nuclei blue by dye lake formation. The eosin azure solution being acidic stains the cytoplasm. The eosin stains the mature cells while light green stains the young cells. Orange G stains the cytoplasm and stains keratin.

### Staining technique

1. Fix the smear in 95%ethanol
2. Hydrate smears through ethanol grades of 80%70%and then 50%
3. Rinse in distilled water 10 dips
4. Stain in Harris haematoxylin for 4 minutes
5. Rinse in tap water.
6. Differentiate in 0.05%acid water 10 dips
7. Rinse in tap water and blue in Scott's tap water 10 dips
8. Rinse in 95%ethanol 10 dips
9. Stain in O.G 6 for 2 minutes
10. Rinse in 95%ethanol 10dips
11. Stain in E.A.50 for 4 minutes
12. Rinse in 95%ethanol 10 dips
13. Dehydrate in changes of absolute ethanol 10 dips each
14. Clear in 3 changes of xylene 10 dips each
15. Mount in D.P.X cover-slip



## APPENDIX VIII: MODIFIED PAP STAINING METHOD

95% alcohol (for fixation)

0.5 % acetic acid	10 dips
Harris's Haematoxylin, preheated 60° C	10 dips
Water	10 dips
0.5 %acetic acid	10 dips
EA-50	10 dips
0.5 % acetic acid	10 dips
95% alcohol	10 dips
Xylene	10 dips
Dpx and mount	cover- slip

### **NB: Results of pap staining**

The full color spectrum is seen as a result of occasional blending of dyes in the same cellular site: red, orange, yellow, green, blue, and violet. These sites are usually colored as described:

- Blue = chromatin
- Yellow to orange = keratinized cells, occasionally erythrocytes, e.t.c.
- Orangish-pink to red = superficial squamous cell cytoplasm, erythrocytes e.t.c.
- Green = cytoplasm of all other cell types

## **APPENDIX IX: The Bethesda System 2001**

### Statement on Specimen Adequacy

Satisfactory for interpretation

Less than optimal

Unsatisfactory

### **Infection**

#### Fungal

Fungal organisms morphologically consistent with *Candida* species

#### Bacterial

Microorganisms morphologically consistent with *Gardnerella* species

Microorganisms morphologically consistent with *Actinomyces* species

Cellular changes suggestive of *Chlamydia* species infection, subject to confirmatory studies

#### Other Protozoan

*Trichomonas vaginalis*

#### Viral

Cellular changes associated with cytomegalovirus

Cellular changes associated with herpesvirus simplex

(Note: for human papillomavirus IHPVJ, refer to "Epithelial Cell

Abnormalities, Squamous Cell")

#### Other

### **Reactive and reparative changes**

#### Inflammation

Associated cellular changes

Follicular cervicitis

Miscellaneous (as related to patient history)

Effects of therapy

Ionizing radiation

Chemotherapy

Effects of mechanical devices (e.g., intrauterine contraceptive device)

Effects of nonsteroidal estrogen exposure (e.g., diethylstilbestrol)

### **Other epithelial cell abnormalities**

#### **Squamous Cell**

- Atypical squamous cells of undetermined significance  
(recommended follow-up and/or type of further investigation:  
specify)
- Squamous intraepithelial lesion (SIL) (comment on presence of  
cellular changes associated with HPV if applicable)

Low-grade squamous intraepithelial lesion, encompassing:

Cellular changes associated with HPV

Mild (slight) dysplasia/cervical intraepithelial neoplasia  
grade 1 (CIN I)

High-grade squamous intraepithelial lesion, encompassing:

Moderate dysplasia/CIN II

Severe dysplasia/CIN III

Carcinoma in situ/CIN III

- Squamous cell carcinoma

#### **Glandular Cell**

- Presence of endometrial cells in one of the following circumstances:

Out of phase in a menstruating woman

In a postmenopausal woman

No menstrual history available

- Atypical glandular cells of undetermined significance  
(Recommended follow-up and/or type of further investigation:  
Specify)

Endometrial

Endocervical

Not otherwise specified

- Adenocarcinoma

Specify probable site of origin: endocervical, endometrial,  
extrauterine

Not otherwise specified

- Other epithelial malignant neoplasm: specify

**Nonepithelial malignant neoplasm: specify**

**hormonal evaluation (applies to vaginal smears only)**

- Hormonal pattern compatible with age and history
- Hormonal pattern incompatible with age and history: specify
- Hormonal evaluation not possible

Cervical specimen

Inflammation

Insufficient patient history



**KENYATTA NATIONAL HOSPITAL**

Hospital Rd. along, Ngong Rd.

P.O. Box 20723, Nairobi.

Tel: 726300-9

Fax: 725272

Telegrams: MEDSUP", Nairobi.

Email: [KNHplan@Ken.Healthnet.org](mailto:KNHplan@Ken.Healthnet.org)

5<sup>th</sup> May 2010

Ref: KNH-ERC/ A/475

Gachie Rose Nyambura  
Dept. of Human Pathology  
School of Medicine  
University of Nairobi

Dear Ms. Gachie

**RESEARCH PROPOSAL: "A COMPARISON OF MODIFIED AND STANDARD PAP STAINING METHODS IN THE ASSESSMENT OF CERVICAL SMEARS AT KENYATTA N.HOSPITAL" (P96/03/2010)**

This is to inform you that the KNH/UON-Ethics & Research Committee has reviewed and **approved** your above revised research proposal for the period 5<sup>th</sup> May, 2010 to 4<sup>th</sup> May, 2011.

You will be required to request for a renewal of the approval if you intend to continue with the study beyond the deadline given. Clearance for export of biological specimens must also be obtained from KNH/UON-Ethics & Research Committee for each batch.

On behalf of the Committee, I wish you a fruitful research and look forward to receiving a summary of the research findings upon completion of the study.

This information will form part of the data base that will be consulted in future when processing related research study so as to minimize chances of study duplication.

Yours sincerely

**PROF A N GUANTAI**  
**SECRETARY, KNH/UON-ERC**

c.c. Prof. K. M. Bhatt, Chairperson, KNH/UON-ERC  
The Deputy Director CS, KNH  
The Dean, School of Medicine, UON  
The Chairman, Dept. of Human Pathology, UON  
The HOD, Records, KNH  
Supervisors: Dr. L Muchiri, Dept. of Human Pathology, UON  
Dr. Ndungu J. R. Dept. of Human Pathology, UON

**UNIVERSITY OF NAIROBI**  
**MEDICAL LIBRARY**