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)

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2010
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: ______________

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: ______________

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

Signature: __________________ Date: ______________
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.

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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.
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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AGC-NOS</td>
<td>Atypical Glandular Cells, Not Otherwise Specified</td>
</tr>
<tr>
<td>ASC-H</td>
<td>Atypical Squamous Cells–Cannot Exclude High-Grade Lesion</td>
</tr>
<tr>
<td>ASC-US</td>
<td>Atypical Squamous Cells–Undetermined Significance</td>
</tr>
<tr>
<td>DPX</td>
<td>Distrene Dibutylphalate Xylene</td>
</tr>
<tr>
<td>EA</td>
<td>Eosin Azure</td>
</tr>
<tr>
<td>ECA</td>
<td>Epithelial Cell Abnormality</td>
</tr>
<tr>
<td>FNA</td>
<td>Fine Needle Aspiration</td>
</tr>
<tr>
<td>HPV</td>
<td>Human Papilloma virus</td>
</tr>
<tr>
<td>HSIL</td>
<td>High-Grade Squamous Intraepithelial Lesion</td>
</tr>
<tr>
<td>KNH</td>
<td>Kenyatta National Hospital</td>
</tr>
<tr>
<td>Lab</td>
<td>Laboratory</td>
</tr>
<tr>
<td>LSIL</td>
<td>Low-Grade Squamous Intraepithelial Lesion</td>
</tr>
<tr>
<td>MP</td>
<td>Modified Papanicolaou</td>
</tr>
<tr>
<td>MUFP</td>
<td>Modified Ultra Fine Needle Aspiration Papanicolaou</td>
</tr>
<tr>
<td>NILM</td>
<td>Negative for Intraepithelial Lesion or Malignancy</td>
</tr>
<tr>
<td>OG</td>
<td>Orange G</td>
</tr>
<tr>
<td>PAP</td>
<td>Papanicolaou Smear/Test/Conventional Papanicolaou</td>
</tr>
<tr>
<td>REAP</td>
<td>Rapid, Economic, Acetic Acid Papanicolaou Stain</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous Cell Carcinoma</td>
</tr>
<tr>
<td>SP</td>
<td>Standard Papanicolaou</td>
</tr>
<tr>
<td>TBS</td>
<td>The Bethesda System</td>
</tr>
<tr>
<td>UON</td>
<td>University of Nairobi</td>
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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, cytodiagnosis, 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 cytodiagnoses 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. (1) 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. (2)

Cervical cancer is preventable, and screening methods exist to detect it at a precancerous state. Cytology-based Pap smear is one such reliable tool. (3) 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. (4) 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. (5) 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. (6)

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. (1) 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. (8)
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.\(^9\) Epidemiological data show that annual screening reduces the mortality by 70% and the probability of developing invasive carcinoma is reduced by over 95%.\(^{5, 10}\)

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.\(^{11}\) 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.\(^{12}\)

2.3 Cervical cancer Screening

In virtually all population-based screening programs to date, the standard screening method has been the Pap smear.\(^1\) The Papanicolaou test/smear is a screening test used to detect premalignant and malignant (cancerous) processes in the ectocervix.\(^6\) 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. (4)

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. (5)

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. (6)
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). \(^{(13)}\)

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). \(^{(13)}\)

### 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. \(^{(9)}\) HPV testing combined with cytology is a reasonable approach in elderly women in order to increase the screening interval to 3-5 years. \(^{(8)}\)

### 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. \(^{(14)}\) 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 Papanicoloau method.\(^{(15)}\)

2.4 Pap stain modifications

Papanicolaou stain has undergone various modifications in different laboratories.\(^{(16)}\) 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.\(^{(17)}\)

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.\(^{(18)}\)

In this modification; Gill’s haematoxylin, EA-36, and isopropanol were used instead of Richard-Allan haematoxylin, cytostain, 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. 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 Alvalez. This pap staining method was further modified to apply to cervico-vaginal smears. The Yang et al modification of the Yang-Alvarez FNA staining method required 12 separate post-fixing, pre-cover-slip 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., \(^{22}\) 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., \(^{23}\) 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., (16) 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. (17) 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., (22) 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.\(^7\)

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.\(^4\) 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.\(^22\)

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.
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. \(^{(24)}\)

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

\[ n = \frac{Z^2 \cdot p \cdot (1-p)}{d^2} \] \(^{(25)}\)

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 ±5 was considered acceptable.

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

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 cervex 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 cervex 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 cervex broom and the cervex 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.\(^{(26)}\)

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. \(^{(27)}\)

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 wound 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>
<thead>
<tr>
<th>Characteristic (variables)</th>
<th>Numbers (n)</th>
<th>Percent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Previous cervical smear</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>106</td>
<td>65.4</td>
</tr>
<tr>
<td>Yes</td>
<td>56</td>
<td>34.5</td>
</tr>
<tr>
<td>2. Normal or Abnormal Previous Pap test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>51</td>
<td>91.4</td>
</tr>
<tr>
<td>Abnormal</td>
<td>2</td>
<td>3.6</td>
</tr>
<tr>
<td>unknown</td>
<td>3</td>
<td>5.35</td>
</tr>
<tr>
<td>3. History of previous STI (Last 6 months)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>153</td>
<td>94.6</td>
</tr>
<tr>
<td>Yes</td>
<td>9</td>
<td>5.4</td>
</tr>
<tr>
<td>4. History of Smoking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>161</td>
<td>99.4</td>
</tr>
<tr>
<td>Yes</td>
<td>1</td>
<td>0.6</td>
</tr>
<tr>
<td>5. Use of contraceptives</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>69</td>
<td>35.5</td>
</tr>
<tr>
<td>Yes</td>
<td>93</td>
<td>58.4</td>
</tr>
<tr>
<td>5.1 Contraceptives</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>30</td>
<td>18.5</td>
</tr>
<tr>
<td>Natural</td>
<td>9</td>
<td>5.4</td>
</tr>
<tr>
<td>OCP</td>
<td>33</td>
<td>19.9</td>
</tr>
<tr>
<td>Condom</td>
<td>10</td>
<td>6.0</td>
</tr>
<tr>
<td>IUCD</td>
<td>61</td>
<td>32.3</td>
</tr>
<tr>
<td>Injectables</td>
<td>6</td>
<td>3.6</td>
</tr>
<tr>
<td>BTL</td>
<td>6</td>
<td>3.6</td>
</tr>
<tr>
<td>Jadelle</td>
<td>7</td>
<td>4.2</td>
</tr>
<tr>
<td>6. Age at First sexual intercourse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤10</td>
<td>2</td>
<td>1.2</td>
</tr>
<tr>
<td>11-15</td>
<td>9</td>
<td>5.4</td>
</tr>
<tr>
<td>16-20</td>
<td>102</td>
<td>61.4</td>
</tr>
<tr>
<td>&gt;20</td>
<td>52</td>
<td>31.3</td>
</tr>
<tr>
<td>7. Number of sexual Partners (Last 6 months)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>19</td>
<td>11.4</td>
</tr>
<tr>
<td>One</td>
<td>136</td>
<td>84.6</td>
</tr>
<tr>
<td>none</td>
<td>6</td>
<td>3.7</td>
</tr>
</tbody>
</table>
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 milliters 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 $(0.90 + 18.50) = Ksh 19.40$ (Table 2).

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

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

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

![Bar chart showing the percentage of distinct and indistinct nuclear borders in Standard and Modified methods.]

<table>
<thead>
<tr>
<th></th>
<th>Standard</th>
<th>Modified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear borders Distinct</td>
<td>152 (95.6%)</td>
<td>154 (96.9%)</td>
</tr>
<tr>
<td>Nuclear borders Indistinct</td>
<td>7 (4.4%)</td>
<td>5 (3.1%)</td>
</tr>
</tbody>
</table>
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 (× 10)  (ii) Standard Pap (×10)
2. Clue cells


3. High grade squamous intraepithelial lesion

(i) Modified Pap (× 40)  (ii) Standard Pap (× 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)

<table>
<thead>
<tr>
<th>CYTODIAGNOSIS</th>
<th>NUMBER OF CASES</th>
<th>PERCENTAGE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NILM</td>
<td>152</td>
<td>95.6%</td>
</tr>
<tr>
<td>ASCUS</td>
<td>2</td>
<td>1.26</td>
</tr>
<tr>
<td>ASCUS-H</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AGC-NOS</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LSIL</td>
<td>3</td>
<td>1.89</td>
</tr>
<tr>
<td>HSIL</td>
<td>2</td>
<td>1.26</td>
</tr>
<tr>
<td>SCC</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TOTAL</td>
<td>159</td>
<td>100</td>
</tr>
<tr>
<td>Others (inflammation &amp; reactive)</td>
<td>96</td>
<td>60.37</td>
</tr>
</tbody>
</table>

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)
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. (13) 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. (4)

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., (28) 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., (22) 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 (22) 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.\textsuperscript{(22)} 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.\textsuperscript{(16)} 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.,\textsuperscript{(22)} and Roy et al.,\textsuperscript{(16)} 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 at el., (29) 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 (19) and Ultrafast Pap (20), 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 in case of any result dispute.
REFERENCES


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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
A 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 TITLE

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 0   Yes 1

11. History of smoking
   No 0   Yes 1

12. 1 Previous Pap test:
   No 0   Yes 1
   12.2 If yes, Normal 1 Abnormal 2

13. 1 Use of contraceptives No 0   Yes 1
   32.2 If yes specify Natural 0 OCP 1
   Condom 2 IUCD 3 Injectables 4 Others 5

14. 1 History of previous malignancy No 0   Yes 1
   14.2 If yes, specify ...........................................

15. History previous radiotherapy (last 6 months) No 0   Yes 1

16. Cervix examination: Normal 1 Erosion 2 Inflamed 3 Suspicious 4
APPENDIX III: RESULTS FORM

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

STUDY NO. --------------------------------------------------------

Papanicolaou smear

Specimen adequacy (method F)

<table>
<thead>
<tr>
<th>Cytoplasmic staining: Unsatisfactory</th>
<th>Satisfactory</th>
<th>Excellent</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Nuclear borders: Indistinct

<table>
<thead>
<tr>
<th>Chromatin</th>
<th>Hazy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Distinct</td>
<td>2</td>
</tr>
</tbody>
</table>

Interpretation of the results

Comment -----------------------------------------------------------------------------------------

Specimen adequacy (Method R)

<table>
<thead>
<tr>
<th>Cytoplasmic staining: Unsatisfactory</th>
<th>Satisfactory</th>
<th>Excellent</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Nuclear borders: Indistinct

<table>
<thead>
<tr>
<th>Chromatin</th>
<th>Hazy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Distinct</td>
<td>2</td>
</tr>
</tbody>
</table>

Interpretation of the results

Comment -----------------------------------------------------------------------------------------

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

Consensus report (QC) -------  

56
APPENDIX IV: DUMMY TABLES OF THE RESULTS

TABLE (1): RESULTS PER SMEAR

<table>
<thead>
<tr>
<th>METHODS</th>
<th>NORMAL</th>
<th>ASCUS</th>
<th>ASCUC-H</th>
<th>LSIL</th>
<th>HSIL</th>
<th>SCC</th>
<th>AGUS-NOS</th>
<th>OTHERS(specify)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROUTINE PAP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**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): CYTOMORPHOLOGICAL FEATURES

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>SCORES</th>
<th>CONVENTIONAL PAP</th>
<th>%</th>
<th>MODIFIED PAP</th>
<th>%</th>
<th>P VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cell borders</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>2. Cytoplasmic staining</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unsatisfactory</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Excellent</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Nuclear borders</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indistinct</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distinct</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>4. Chromatin</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hazy</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crisp</td>
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</tr>
</tbody>
</table>
TABLE (3): DUMMY TABLE OF CYTODIAGNOSIS

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

The specimen adequacy for scoring will be determined by ≥ 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. Cytocentrifugation 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

![Diagram of Cytospin apparatus](image)

Microtube  cytofix  cytobrush

Slide(smear area)

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

Lithotomy position  speculum  ectocervix  endocervix

Source: Spears/Gillette 2010
APPENDIX VII: STANDARD PAPANICOLOAU STAINING METHOD

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 (appues 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
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 5th May, 2010 to 4th 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

cc: 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