Assessment of Staining Quality in the Cytology Smears Using Papnicolaou, Diff-Quik and Hematoxlyine and Eosin Stains, Faculty of Medical Laboratory Sciences, University of Gezira, Sudan (2017)

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(B.Sc in Histopathology and Cytopathology, University of Gezira 2014)

A Dissertation
Submitted to University of Gezira in Partial Fulfillment of The Requirement for The Award of the Degree of Master of Sciences in Histopathology and Cytopathology

Histopathology and Cytopathology Department
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Date: 3 / 11 /2017
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Date:  3 / 11 /2017
DECLARATION

This thesis is a presentation of my original research work, wherever contributions of others are involved, and every effort is made to indicate this clearly, with due to reference to the literature, and acknowledgment of collaborative research and discussion. The work was done under the guidance of Dr. Dr. Abd Alrheem Ali Babker and Prof. Bakri Yousif Mohammed at University of Gazira, Faculty of Medical Laboratory sciences.

Huda Albagir Altayib Abdallah
Dedication

Every challenging work needs self efforts as well as guidance of elders especially those who were very close to our heart.

My humble effort I dedicate to my sweet and loving

Father & mother,

Whose affection, love, encouragement and prays of day and night make me able to get such success and honor,

Along with all hard working and respected

Teachers
Acknowledgement

Above all, I would like to thank of Allah I express my sincere gratitude to the main Supervisor Dr. Abd Alrheem Ali Babker for continued support for my research, for his patience, motivation, enthusiasm, and tremendous knowledge. His guidance helped me all the time in research and writing this theses. And I thank the co-supervisor Prof. Bakri Yousif Mohammed Also all the workers in the medical laboratory department of histopathology and cytopathology and all those who contributed to the completion of my research Last, I would like to thank my family.
Assessment of Staining Quality in the Cytology Smears Using Papnicolaou, Diff-Quik and Hematoxlyine and Eosin Stains, Faculty of Medical Laboratory Sciences, University of Gezira, Sudan (2017)

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Abstract

Cytology is the art and science of the interpretation of cells removed from the human body through clinical procedures or exfoliation. The most widely used stains in cytology are Papanicolaou stain, Diff-Quik stain, and Haemotoxylin and Eosin stain. This descriptive cross sectional study was conducted in the faculty of medical laboratory science department of histopathology and cytopathology from April - July 2017. The study aimed to find the best stain for the demonstration of cytological smears. 120 samples were collected randomly from different cytology samples and divided equally (urine, sputum, Fine Needle Aspiration and buccal smear) and stained by Papanicolaou, Diff-Quik and Hematoxlyine and Eosin stains. The result showed that the nucleus, cytoplasm and general cytological appearance in Fine Needle Aspiration smears were excellently stained by Papanicolaou stain followed by Diff-Quik stain followed by Hematoxlyine and Eosin stain. showed that the nucleus, cytoplasm and general cytological appearance in sputum smears were excellently stained by Papanicolaou stain followed by Diff-Quik stain followed by Hematoxlyine and Eosin stain, showed that the nucleus, cytoplasm and general cytological appearance in urine smears were excellently stained by Papanicolaou stain followed by Diff-Quik stain followed by Hematoxlyine and Eosin stain and showed that the nucleus, cytoplasm and general cytological appearance in buccal smears were excellently stained by Papanicolaou stain followed by Diff-Quik stain followed by Hematoxlyine and Eosin stain. The study found that the best stain for the demonstration of Fine Needle Aspiration, sputum, urine and buccal smears is Papanicolaou stain followed by Diff-Quik stain and Hematoxlyine and Eosin stain. The study recommends the use of Papanicolaou stain as routine stain for cytological smears.
تقيم جودة الأصباغ في مسحات الخلوية باستخدام أصباغ بابانيكولاو، ديف-كويك و هيماتوكسلين والإيوسين. كلية علوم المختبرات الطبية جامعة الجزيرة، السودان (2017)

هدى الباقر الطيب عبده الله

ملخص الدراسة

علم الخلايا هو فن وعلم تفسير الخلايا التي إزيلت من جسم الإنسان من خلال الإجراءات السريرية أو تشخيص.

الأصباغ الأكثر استخداماً على نطاق واسع في علم الخلايا هي أصباغ بابانيكولاو، ديف-كويك و هيماتوكسلين والإيوسين. أجريت هذه الدراسة الوصفية المقطعة في كلية علوم المختبرات الطبية قسم الأنسجة والخلايا المريضة من أبريل - يوليو 2017. هدفت الدراسة إلى العثور على أفضل صبغة لإظهار مسحات الخلوية. تم جمع 120 عينة عشوائياً من عينات علم الخلايا المختلفة وقسمت بالتساوي (البول، والتفاف، الرشف بالإبر الناعمة وتشويه الشدق) وصبغت بصبغة بابانيكولاو، ديف-كويك و هيماتوكسلين والإيوسين. وأظهرت النتائج أن الأصباغ الأكثر استخداماً هي اصباغ بابانيكولاو، ديف-كويك و هيماتوكسلين والإيوسين. أظهرت النتائج أن النواة والهيل والمنطقة الخلوية العامة في مسحات الرشف بالإبر الناعمة صبغت بشكل ممتاز بصبغة بابانيكولاو. تليها صبغة ديف-كويك و تليها صبغة هيماتوكسلين والإيوسين. و أظهرت أن النواة والهيل والمنطقة الخلوية العامة في مسحات التفاف صبغت بشكل ممتاز بصبغة بابانيكولاو تليها صبغة ديف-كويك و تليها صبغة هيماتوكسلين والإيوسين. وأظهرت أن النواة والهيل والمنطقة الخلوية العامة في مسحات البول صبغت بشكل ممتاز بصبغة بابانيكولاو تليها صبغة ديف-كويك و تليها صبغة هيماتوكسلين والإيوسين. و أظهرت أن النواة والهيل والمنطقة الخلوية العامة في مسحات تشويه الشدق صبغت بشكل ممتاز بصبغة بابانيكولاو تليها صبغة ديف-كويك و تليها صبغة هيماتوكسلين والإيوسين. و وجدت الدراسة أن أفضل صبغة لإظهار مسحات الرشف بالإبر الناعمة و مسحات التفاف ومسحات البول ومسحات تشويه الشدق هي صبغة بابانيكولاو تليها صبغة ديف-كويك و صبغة هيماتوكسلين والإيوسين. و توصي الدراسة باستخدام صبغة بابانيكولاو كصبغة روتينية لصبغ الخلايا.
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Chapter One

Introduction

Cytology is the study of cells from the body. These cells are obtained by either spontaneous exfoliation into a body fluid or by physical exfoliation. Cells are prepared onto glass slides and stained to allow for visualization using the light microscope. The role of the scientist is to prepare and examine these slides to detect and differentiate pre-malignant and malignant conditions from benign entities. They work with cytopathologists to provide a diagnosis.

Cytology is a challenging and rewarding career and offers a hands-on approach to medical testing. (www.otago.ac.nz/)

Diagnostic cytology is the science of interpretation of cells that are either exfoliated from epithelial surfaces or removed from various tissue. George N Papanicolaou introduce cytology as a tool to detect cancer and pre-cancer in 1928. It is now widely accepted method for mass screening in asymptomatic population. Many European countries have achieved reduction in incidence of cervical cancer by systemic pap smear screening of the population.

The advantage of diagnostic cytology are that it is a non-invasive, simple procedure, helps in faster reporting, is relatively inexpensive, has high population acceptance and facilitates cancer screening in the field. Diagnostic cytology can be carried out by different method, which includes collection and examination of exfoliated cells such as vaginal scrapes, sputum, urine, body fluids etc. Collection of cells by brushing, scraping or abrasive techniques is usually employed to confirm or exclude malignancy. Fibreoptic endoscopes and other procedures can be used for collecting samples directly from the internal organs.

Fine-Needle Aspiration Cytology/ Biopsy(FNAC/FNAB) is now a widely accepted diagnostic procedures, which has largely replaced open biopsy. This method is applicable to lesions that are easily palpable, for example swelling in Thyroid, Breast, superficial Lymph node etc. Imaging techniques, mainly ultra-sonography and computed tomography, offer an opportunity for guided FNAC of deeper structure.

The practice of diagnostic cytology needs proper training of the laboratory personnel including cytopathologist, cytotechnologist and cytotechnician. The role of
cytotechnician is very important in cancer control programmes where large numbers of asymptomatic population have to be screened.

The accuracy of the cytological examination from any body site depends greatly on the quality of collection, preparation, staining and interpretation of the material. Inadequacy in any of these steps will adversely affect the quality of diagnostic cytology.

Diagnostic accuracy and reliability are major issues in cytology practice. Over the years many quality control measures have been introduced for ensuring high standards in cytology. Among them the most important are regular continuing education of medical and technical personnel, certification and accreditation of laboratory to national authorities such as Indian Academy of Cytologist(IAC), introduction of quality assurance and quality control measures, computerization, introduction of internationally accepted terminology, improvement of sample preparation techniques, quantitative and analytical cytology techniques and advanced technologies including automation.(Leopold G. Koss, 1979),(George L. Wied, 1997)

**Cytology stains:**

Cytology is the art and science of the interpretation of cells removed from the human body through clinical procedures or exfoliation. One of the most widely used groups of stains in cytology.

**Papanicolaou Staining :**

Papanicolaou staining method is the routine staining procedure used in cytopathology laboratory. This technique is named after Dr. George N. Papanicolaou, the father of exfoliative cytology and is devised for the optimal visualization of cells exfoliated from epithelial surfaces of the body. It is a polychrome staining reaction designed to display the many variations of cellular morphology showing degree of cellular maturity and metabolic activity. The use of the Papanicolaou stain results in well stained nuclear chromatin, differential cytoplasmic counterstaining and cytoplasmic transparency.(Carson, Freida L., Hladik, Christa, 2009)
**Haematoxylin and Eosin (H&E) staining:**

Some laboratories use routine H&E stain non-gynecological smears. The benefits of using Papnicolaou stains are clear definition of nuclear details and differential counter staining giving cytoplasmic transparency. H&E stain does not satisfy these criteria and hence unacceptable for cervical smears. (Claude gompel, John Wiley, Sons 1978)

**Diff-Quik staining:**

is a commercial Romanowsky stain variant, commonly used in histological staining to rapidly stain and differentiate a variety of smears, commonly blood and non-gynecological smears, including those of fine needle aspirates. It is based on a modification of the Wright Giemsa stain pioneered by Bernard Witlin in 1970. It has advantages over the older Wright Giemsa staining technique, as it reduces the 4 minute process into a simplified 15 second operation, and allows for selective increased eosinophilic or basophilic staining depending upon the time the smear is left in the staining solutions. (Demay. November 2006)
1.2. Justification and objectives:

1.2.1 Justification:
- Needing for better staining and satisfy for diagnosis smear cytology.
- Because use of the Papanicolaou stain result in well stained nuclear chromatin, differential cytoplasmic counterstaining and cytoplasmic transparency.
- Use the Diff-Quik stain rapidly and differentiate a variety of smears.
- Use the Haematoxylin & Eosin stain because fast in action and stable for at last year.

1.3 Objectives:

1.3.1 General objectives:
To identify assessment of staining quality in the cytology smears using Papnicolaou, Diff-Quik and Hematoxyline & Eosin stains, faculty of medical laboratory sciences University of Gezira, Gezira State, Sudan (2017)

1.3.2 Specific objectives:
- To compare between Papnicolaou stain and Diff-Quik stain in the Fine Needle Aspiration smear and sputum smear and buccal smear.
- To compare between panicolaou stain and Haemotoxylin&Eosin stain in the Fine Needle Aspiration smear and urine smear.
- To find out the better stain in diagnosis of cytology.
Chapter Two

Literature reviews

2.1 Cytology:
Is the study of cells (Bethesda, MD, 2004). Cytology is that branch of life science that deals with the study of cells in terms of structure, function and chemistry. Robert Hooke (1635 – 1703) is sometimes seen as the father of cytology. Based on usage it can refer to:

- Cytogenetics.
- Cell biology: the study of (normal) cellular anatomy, function and chemistry.

The international Academy of cytology has as its official journal Acta Cytologica.

2.2 History:
Cells, that were once invisible to the naked eye, became visible in 17th century Europe with the invention of the compound microscope. Robert Hooke was the first person to term the building block of all living organism as "cells" after looking at cork (Hooke, Robert, September 1665). The cell theory states that all living things are made up cells (Gupta, Dec 1, 2005). The theory also states that both plant and animal scientist, Matthias Schleiden and animal scientist, Theodor Schwann in 1839 (Gupta, Dec 1, 2005). 19 years later, Rudolf Virchow contributed to the cell theory, arguing that all cells come from the division of preexisting cells (Gupta, Dec 1, 2005). In recent years, there have been many studies which question the cell theory. Scientists have struggled to decide whether viruses are alive or not. Viruses lack common characteristics of a living cell, such as membranes, cell organelles and the ability to reproduce by themselves (Kendrick, Karolyn, Jan 1, 2010). Viruses range from 0.005 to .03 microns in size whereas Bacteria range from 1 – 5 microns (Cullimore, D., Dec 17, 2007). The late 19th century indicates the birth of cytology. Modern day cell biology research looks at different ways to culture and manipulate cells outside of a living body to further research in human anatomy and physiology to derive treatments and other medications, etc. The techniques by which cells are studied
have evolved. Advancement in microscope techniques and technology such as
fluorescence microscopy, phase-contrast microscopy, dark field microscopy,
confocal microscopy, cytometry, transmission electron microscopy, etc.
have allowed scientists to get a better idea of the structure of cells(Gupta, Dec 1,
2005).

2.3 Cell structure:
Fundamental classification of cells: prokaryotes and eukaryotes. The major difference
between the two is the presence and/or absence of organelles. Other factors such as
size, the way in which they reproduce, and the number of cells distinguish them from
one another(Doble, Mukesh; Gummadi, Sathyanarayana N.,August 5, 2010).
Eukaryotic cells include animal, plant, fungi and protozoa cells which all have a
nucleus enclosed by a membrane. Prokaryotic cells, lacking an enclosed nucleus,
include bacteria and archaea. Prokaryotic cells are much smaller than eukaryotic cells,
making prokaryotic cells the smallest form of life(Kaneshiro, Edna ,May 2, 2001).
Cytologists typically focus on eukaryotic cells whereas prokaryotic cells are the focus
of microbiologists, but this is not always the case.

2.4 Branches:
These are the main branches of cytology(Pratiyogita Darpan. June 1999):
- Cytotaxonomy – The study combining cytology and taxonomy.
- Cytogenetics – The study combining cytology and genetics.
- Cell Physiology - The study combining cytology and physiology.
- Cytochemistry – The study combining cytology and chemistry.
- Cytopathology – The study combining cytology and pathology.
- Cytoecology - The study combining cytology and ecology.

2.5 Information is obtained by cytology:
The most important thing cytology can tell us is whether a problem is caused by
inflammation or by neoplasia (literally "new growth"). If there is inflammation,
cytology can often identify an underlying cause, such as bacterial infection,
embedded foreign body, or allergies. If the sample appears to be neoplastic,
cytology can usually determine which type of tissue is involved, and whether the
neoplasm is malignant (cancer) or benign.( Kristiina Ruotsalo, December 2, 2008)
2.6 Collection and Preparation of Material for cytodiagnosis:

Accurate interpretation of cellular material is depended on the following factors:

- Method of specimen collection.
- Fixation and fixative.
- Preservation of fluid specimen prior to processing.
- Preparation of material for microscopic examination.
- Staining and mounting of the cell sample.

2.6.1 Methods of specimen collection:

Individual cells may be studied in many way.

A. Exfoliative Cytology: it is the study of cells that have been shed or removed from the epithelial surface of various organ. Cells from organ, which communicate with the exterior of the body, are suitable for study. These cells can be recovered either from natural secretions such as urine, sputum and vaginal or prostate fluids or by artificial means such as paracentesis or lavage. The cells can be collected from the epithelial surfaces by lightly scraping the surface, by swabbing, aspirating or washing the surfaces. Normal cells are cohesive in nature but exfoliated when they attain maturation. During malignant conditions or during infection, the exfoliation becomes exaggerated and the epithelial cells show variation in morphology. Such exfoliated cells, when collected and appropriately stained, give information on the living epithelium from which they are derived. These characteristic cellular and nuclear appearance in cells thrown off from healthy epithelium, differ distinctly from those, derived from inflamed or malignant lesion. Thus by studying the alterations in morphology of the exfoliated cells and their pattern, the diagnosis of various pathological conditions can be made. (Richard M., 1996).

B. Fine Needle Aspiration Cytology (FNAC): This is a technique used to obtain material from organs that do not shed cells spontaneously. It is valuable in diagnosis of lesions of the breast, thyroid, lymph node, liver, lungs, skin, soft tissues and bones. (Richard M., 1996).

C. Body Fluid: Body fluid like Urine, Pleural fluid, Pericardial fluid, Cerebrospinal fluid, Synovial fluid and Ascitic fluid can be studied by cytology.
2.6.1.1 EXFOLIATIVE CYTOLOGY:

2.6.1.1.1 Female Genital Tract (FGT)

The cytological specimens collected from FGT include cervical smear, vaginal smear, aspiration from posterior fornix of vagina (vaginal pool smear) and endometrial smear. (Erica G. Watchel, 1964)

**Cervical smear:** Cancer of the uterine cervix is the commonest cancer in the FGT. Almost all invasive cancers of the cervix are preceded by a phase of preinvasive disease. Early detection even at the preinvasive stage is possible by doing cervical smear (Pap Smear Test). This can identify patients who are likely to develop cancer and appropriate interventions may be carried out. (Dinae Soloman, 2003)

**Advantage of Pap smear:**

- It is painless and simple.
- Does not cause bleeding.
- Does not need anesthesia.
- Can detect cancer and precancer.
- Can identify non-specific and specific inflammations.
- Can be carried out as an outpatient procedure.

**Patient preparation:** proper patient preparation is the beginning of good cervical cytology. The patient should be instructed before coming for smear collection, that she should not douche the vagina for at least a day before the examination. No intravaginal drugs or preparation should be used for at least one week before the examination and the patient should abstain from coitus for one day before the examination. Smear should not be taken during menstrual bleeding, because of contamination with blood, endometrial component, debris and histiocytes. (Erica G. Watchel, 1964)

**Sampling:** A cervical cytological sample is considered satisfactory for cytological diagnosis when their composition reflects the mucosal lining of the cervix, encompassing ectocervical, squamous metaplastic cells and endocervical columnar cells in fair numbers. It is generally agreed that majority of epithelial abnormalities that eventually lead to an invasive cancer originate in the squamo-columnar junction (transformation zone). As stated by the British Society for Clinical Cytology (BSCC),
a cervical smear if properly taken should contain cells from the whole transformation zone (TZ). The sample should contain a sufficient quantity of epithelial cells, and both metaplastic and columnar cells should be present. According to the Bethesda System, an adequate smear contains an adequate endocervical/transformation zone component. Lubricant should not be used while examining, as it can obscure the cells during smear examination. (Erica G. Watchel, 1964)

**Factor effecting specimen collection:** The experience of the person who is taking the smear is very important in getting smears with adequate cellular composition. Clinicians must receive appropriate training in taking cervical scrape samples and slide preparation. The cervix must be clearly visualized and the entire transformation-zone is scraped. It is also the responsibility of sample takers and quality assurance programmes to monitor the quality of specimens, so as to minimize / avoid inadequate samples and preparation / fixation artifacts. Periodic feedback to clinicians regarding the quality of their samples is important in this regard. (Erica G. Watchel, 1964)

**Sampling Devices:** The collection device may play an important role in sample adequacy. The shape, surface, texture and material of the device may determine how much of the scraped material is deposited on to the glass slide and is available for screening and analysis. Several methods of obtaining cytologic material from the uterine cervix are available. However, use of cotton swab for collection of cervical smear is to be discouraged, in view of the drying artifacts and loss of cells, which are caused by this method. (Erica G. Watchel, 1964)

- Smears obtained with original Ayre's spatula are often easier to screen. Wooden spatula is preferable to plastic spatula, because of its mildly rough surface that can collect more material. The disadvantages are that the method may occasionally be traumatic to the patient, and the tip of spatula that does not fit the external os may fail to remove some of the valuable material from the squamo-columnar junction.
- Based on the original wooden Ayre's spatula, many devices of different shapes and sizes have been introduced to improve sampling. This includes Endocervical Brush, Cervex, Cytobrush, etc.
- The pointed Aylesbury version of cervical spatula was designed to sample cells from both endocervix and the transformation zone (TZ) of the cervix.
- The cervex brush device is a flexible plastic brush, which follows the shape of the endocervix, transformation zone and ectocervix as well and is suitable for every cervix shape.
- Endo-cervical brush is a small bottlebrush like device with one end having fine bristles made up of nylons. This device is strictly for taking materials from endocervix. Gently insert the brush in endocervix and rotate one turn pressing in the upper and lower wall.
- The cytobrush is similar to that of endocervical brush except that the projected tip is without bristles. This can be used for obtaining cells from the whole cervix.

Single sampling devices and methods have their limitations in obtaining adequate smears from the cervix. A combination of two devices, usually spatula and endocervical brush, give better result. Triple smear or the vaginal-cervical-endocervical (VCE) technique can provide the best result. However, feasibility and cost factor need to be taken into consideration.

In postmenopausal women, the squamo-columnar junction recedes making it difficult to obtain good amount of endocervical cells and cells from TZ. Hence a combination of two devices, spatula plus endocervical brush preferred. In those with a prolapsed uterus, the cervix is first soaked with normal saline and scrape is collected with cytobrush. To obtain a satisfactory smear from a bleeding cervix, the blood is wiped with wet cotton and smear is obtained by wooden spatula.

There has been some concern that the use of the endocervical brush can result in the appearance of a much greater number of endocervical cells in a smear and that their arrangement in large sheets might mimic malignancy. To avoid this problem clinicians should inform the laboratory when an endocervical brush is used for collecting the smear.

**Preparation of smear:** After smear collection, the cellular sample is evenly smeared on to the center of the non-frosted area of the glass slide, by rotating both slides of the scrape end of the spatula in multiple clockwise swirls in contact with the slide end
fixing it immediately. Excessively thin or thick smears can result in false-negative reports. The smear should be visually inspected after fixation. If it does not appear satisfactory, repeat it during the same examination and submit both slides for cytological examination.

Some studies have shown that two-slide cervical smears detect more abnormalities than a one-slide smear. Two smears do increase screening cost over a single-slide smear, but those costs are not double that of a single-slide examination. A two-instrument collection on a single slide increase screening time only minimally over a single instrument. (Erica G. Watchel, 1964)

**Vaginal smear:** Introduce an unlubricated speculum, scrape the lateral vaginal wall at the level of cervix with a spatula. The broad and flat end of Ayre's spatula is used for this purpose. The cellular material is rapidly but gently smeared on a clean glass slide and the smears fixed immediately. If no spatula is available a cotton swab dipped in normal saline can be used. (Erica G. Watchel, 1964)

**Vaginal pool smear:** The aspiration can be performed after the introduction of unlubricated speculum. The technique allows collection of cells under direct vision from posterior fornix pool. When a speculum is not employed the pipette is gently introduced in to the vagina until resistance is encountered. It is important to compress the suction bulb during the introduction of the pipette to avoid collecting the cellular material of the lower vaginal origin. The cellular material is spread on a clean glass slide and fixed immediately. (Erica G. Watchel, 1964)

**Endometrial aspiration smear:** After preliminary visualization and cleaning of cervix a sterile cannula is introduced into the uterine cavity and aspiration is then carried out with a syringe. The specimen is squirted on a clean glass slide, gently spread and rapidly fixed. (Erica G. Watchel, 1964)

### 2.6.1.1.2 Respiratory Tract

Respiratory tract malignancies can be detected mainly by sputum cytology or by bronchoscopy material.

**Sputum cytology:** sputum specimen can be obtained from the patient either spontaneously or by aerosol – induced method. Morning specimen resulting from overnight accumulation of secretion yields best result. Three to five consecutive day's
sputum samples should be examined to ensure maximum diagnostic accuracy. Fresh unfixed specimens are better than prefixed specimen in 70% ethyl alcohol or coating fixative such as carbowax or saccomano fixative. The sputum must be carefully inspected by pouring the specimen into a petri dish and examining on a dark background. Select any bloody, discolored or solid particles, if present, place a small portion of each particle on a micro slide, spread evenly and fix it immediately. Prefixed specimens should be smeared on albumen or polylysine coated slides. (Richard M, DeMay.1996)

**Bronchoscopic Specimens:** Specimens that are obtained by bronchoscopy are secretions (bronchio-alveolar lavage), direct needle aspirate from suspicious area and bronchial brushing and washings. Post bronchoscopic sputum is one of the most valuable specimens for the detection of pulmonary lesion. (Richard M, DeMay.1996)

### 2.6.1.1.3 Other sites

**Oral lesion:** Scrape the lesion with a tongue depressor, spread material on a clean slide and fix immediately. (Richard M, DeMay.1996)

**Nasopharynx:** Cotton tipped applicator is used to obtain material for cytological examination. (Richard M, DeMay.1996)

**Larynx:** A cotton smear of larynx may be a useful adjunct to clinical diagnosis if biopsy is not contemplated. (Richard M, DeMay.1996)

**Oesophagus:**

Oesophageal washing and brushing are usually recommended for collecting cytology sample from oesophagus. To collect a good specimen for cytology on should first localize the suspicious lesion by oesophagoscopy. (Richard M, DeMay.1996)

**Stomach:**

cytology specimen can be collected from the surface of the lesion by scraping under direct vision of a flexible endoscope. The cells collected can be directly smeared on a glass slide. Gastric lavage is also recommended for cytological investigation. (Richard M, DeMay.1996)
Discharge from nipple of the breast:
Spontaneous nipple discharge and discharge produced by breast massage are collected by applying the slide directly to the nipple followed by immediate fixation. (Richard M, DeMay. 1996)

2.6.1.2 FINE NEEDLE ASPIRATION CYTOLOGY (FNAC)

Procedure, Preparation, and Preservation:
FNAC is the study of cellular samples obtained through a fine needle under negative pressure. The technique is relatively painless and inexpensive. When performed by well-trained pathologists / surgeons / clinicians and reported by experienced pathologists, it can provide unequivocal diagnosis in most of the situations.

It is useful in lesions that are easily palpable, like growth of skin, subcutaneous soft tissue tumors, thyroid, lymph nodes, salivary glands and breast. Guided aspiration by internal imaging techniques like C.T or ultrasonography allows FNA of lesions of internal organs like lung, mediastinum, abdominal and retroperitoneal organs, prostate etc. The low risk of complications allows it to be performed as an out-patient procedure. It is highly suitable in debilitated patients, multiple lesions and easily repeatable. (Svantle R. Orell, 1992)

The three pre-requisites for a meaningful diagnosis on FNAC are:
1. Proper technique – procedure, preparation of smears, fixation, staining.
2. Microscopic evaluation of smears.
3. Correlation of morphology with the clinical picture.

The technique: Attention to technique is necessary to optimize the yield of the sample, making its interpretation easier and more reliable. Expertise regarding the technique comes from constant practice and correlation of the smear technique with the results. (Svantle R. Orell, 1992)

Equipment: The success or failure of the aspiration procedure depends to some extent on the organization of the set up. Some institution set aside appropriately equipped areas dedicated to the procedure. Otherwise, the material can be arranged on movable carts or even in portable containers. Thus FNA can be performed as an outpatient procedure or at the patient's bedside. (Svantle R. Orell, 1992)
**Needles:** Standard disposable 22-24 gauge 1-1.5inch needles are used for plain FNAC. The length and caliber of the needle should fit the size, depth, location and the consistency of the target. For small subcutaneous lesion, one-inch 23 gauge needle is ideal while for a deep-seated breast lesion, longer and larger needle is required. Finer needles are also recommended for children and for vascular organs like thyroid. (Svantle R. Orell, 1992)

**Syringes:** Standard disposable plastic syringes of 10ml are used. Syringe should be of good quality and should produce good negative pressure. 5cc syringes can be used for vascular organs like thyroid. One important factor is to check the tight fit of the needle on the syringe tip. A loosely fitting needle can render the procedure useless and may injure the patient.

Syringe holder: A syringe piston handle can be used, leaving one hand free to immobilize the lesion. This is not absolutely essential and is a matter of choice of the aspirator. (Svantle R. Orell, 1992)

**Slides:** Plain glass slides of good quality are used. Slides should be clean, dry, transparent and grease free. (Svantle R. Orell, 1992)

**Fixative:** 95% ethyl alcohol is recommended. Fixative is kept ready in Coplin jars.

**Other supplies:** Test tubes, pencil for marking, alcohol, swabs for skin, watchglass, saline, adhesive dressing, gloves etc. are needed. All the materials required are assembled in advance before starting the procedure. This is extremely important as delay in fixation can make interpretation of smears difficult. (Svantle R. Orell, 1992)

**Aspiration procedure:**

**Steps to be followed before performing the aspiration** (Richard M. DeMay, 1996)

1. Relevant history and clinical details, radiological findings, provisional diagnosis etc.
2. Lesion to be aspirated is palpated and its suitability for aspiration assessed.
3. The procedure must be clearly explained to the patient and consent and cooperation ensured.
4. Before starting the procedure, ensure that all required equipment, instruments and supplies are available.
5. All universal precautions should be followed during the procedure.
Steps to be performed in the actual performance of the aspiration: (Richard M.DeMay.1996)

- Position the patient.
- Immobilization of the lesion.
- Penetrating the lesion.
- Creation of a vacuum and obtaining the material.
- Release of vacuum and withdrawal of the needle.

Preservation and processing of smears:

There are two fundamental methods of processing smears obtained by FNA. Smears are prepared and fixed according to the requirements of the stain to be used. (Richard M.DeMay.1996)

1. Air-drying followed by hematological stains like May – Grunwald – Giemsa(MGG), Diff Quik stain, Giemsa etc.: In this method, smears are intentionally air dried, but if smears are not correctly made and dried quickly artifacts will result. One advantage is the speed with which smears can be stained especially with use of rapid stains like Diff Quik (2-3 minutes). Rapid stains are particularly useful in preliminary assessment of adequacy of the sample before the patient is released. Colloid, mucin, endocrine cytoplasmic granules etc are better brought out in air-dried preparation. It is also useful in patients with hematological malignancies like lymphoma or leukemia.

2. Alcohol fixation followed by Papanicolaou (Pap) or hematoxylin and eosin (H&E) staining: Rapid fixation in alcohol (wet fixation) is essential for pap staining, which brings out nuclear details clearly, allowing better identification of malignant cells. It also allows better comparison with histology and hence is favored by majority of pathologists. But if the smears are not quickly made and fixed, drying artifact can occur in which case, the cytoplasm takes up more eosin and nuclear details are less clear. A cellular sample can be unfit for diagnosis if there is significant drying. Hence with pap staining, air-drying is avoided as much as possible especially by dropping the slides into the fixative immediately after the smears are made. Poor quality of preparation, fixation or
staining can all make a cellular sample unsatisfactory for evaluation. Hence great care must be taken in preparation and fixation of smears.

2.6.1.3 BODY FLUIDS

**Urine:** For cytological evaluation of bladder, three morning samples of urine (each of 50 – 100 ml) obtained on consecutive days are recommended. Centrifuge the urine for 10 minutes and place one or two drops of sediment on a glass slide, spread the material and fix immediately. Catheterised samples are also acceptable.(Leopold G. Koss. 1979)

**Cerebrospinal fluid (CSF):** CSF and other fluid of small volume have considerable bearing on diagnostic accuracy, the larger the sample the better the result. If several samples are obtained the second or third should be used for cytology. The addition of an equal amount of ethyl alcohol to the CSF is recommended if a delay in processing is anticipated. Considering the low volume and cellularity, CSF specimen should be processed by cytocentrifugation.(Leopold G. Koss. 1979)

**Cytocentrifugation:** The fluid samples with low cell content such as CSF and urine are centrifuged in Cytospin where the cells are sedimented directly on the microslides.(Leopold G. Koss. 1979)

**Preservation of fluid specimens prior to processing:**

Preservation of cellular morphology until the sample can be processed is essential for accurate cytological interpretation. Specimens may be sent to the laboratory without preservation / prefixatives, if facilities for immediate processing are available. The duration between collection and preparation of the sample before cellular damage occur depends on PH, protein content, enzymatic activity and the presence or absence of bacteria. It is not possible to predict these variables even in specimens from the same anatomic site. The following guidelines are useful to get acceptable result.(Leopold G. Koss. 1979)

a. **Specimens with high mucus content** such as sputum, bronchial aspirates, mucocele fluid can be preserved for 12 to 14 hours if refrigerated. Refrigeration slows down the bacterial growth, which
causes cellular damage. Mucus apparently coats the cells, protecting them against rapid degeneration. The cells in specimens diluted with saliva are not as well protected and may deteriorate more rapidly.

b. **Specimens with high protein content** such as pleural, peritoneal or pericardial fluids can be preserved for 24 to 48 hours with refrigeration. The protein-rich fluid in which the cells are bathed acts as a tissue culture medium in preserving cellular morphology.

c. **Specimens with low mucus or protein content** such as urine or CSF will be preserved for 1-2 hours even if refrigerated. The fluid medium in which these cells are bathed contains enzymatic agents capable of causing cell destruction. Refrigeration may inhibit bacterial growth but does not protect the cells.

d. **Specimens with low PH** such as gastric material, must be collected on ice and be processed within minutes of collection to prevent cellular destruction by HCL.

2.7 **Cytopreparatory Techniques of Serous Effusions:**
The term serous effusion refers to the fluid accumulated in the three serous cavities namely pleural, pericardial and peritoneal. It forms an important source of useful diagnostic information in clinical practice. Certain benign processes like florid tuberculosis or rheumatoid pleurisy can be recognized cytologically but the most important goal of effusion cytology is the recognition of malignant cells. For an accurate cytologic diagnosis of serous effusions, attention to proper technique is of paramount important (Stanley Lawrence Lamberg, 1978). The essential requirement are:

- Freshly tapped specimen.
- Immediate processing.
- Rapid fixation of slides.

2.7.1 **Collection and preservation**
pleural, pericardial and peritoneal fluids can be collected in tubes or syringes that may be either plain or pre–heparinised, to prevent coagulation. Cells in heparinised fluids do not deteriorate rapidly and there are some advantages in the processing of these
fluids like layering of many malignant cells in the buffy-coat of the centrifuged sample and better adherence of the cells to the slides. Freshly tapped specimens are preferred for cytology, if facilities for immediate processing are available. If immediate processing is not possible, it can be preserved in the refrigerator for a period of 24-48 hours. Preservation of cells by pre-fixation in 50% ethanol is also possible. Pre-fixation and spray fixatives are recommended when sample has to be sent to a distant laboratory. Albuminized slides should be used to prepare smears from prefixed sample. 20-30ml fluid is generally sufficient to get enough cells for cytological evaluation. If the entire specimen tapped cannot be sent to the laboratory, a representative sample from the bottom part of the fluid should be sent to the laboratory. (Stanley Lawrence Lamberg, 1978)

2.7.2 Gross Examination:
When the specimen is received in the laboratory, the gross appearance and the amount of fluid received are noted down. The fluid may be clear, transparent, straw coloured, yellow, brown, red, chylous, purulent, mucoid or hemorrhagic. The appearance of the fluid also helps in diagnosis. (Stanley Lawrence Lamberg, 1978)

2.7.3 Processing:
2.7.3.1 Routine Processing
The fluid received is stirred briskly to disperse the suspended cells. A representative volume of the fluid (10-15ml) is centrifuged at 2500 rpm for 5 minutes. If possible glass tube should be avoided because of the disadvantages like tendency for cells to adhere to glass and possible breakage. The centrifuge tube must be meticulously cleaned to ensure a perfectly clean inner surface. If the quantity of fluid is too little for centrifugation, an equal amount of normal saline can be added before centrifugation. If fibrin clot has already formed, the clot may be smashed against the sides of the tube by using an applicator and if large clot remains, may be processed as cellblock. Place one to two drops of the sediment on the slide and allow it to spread evenly by placing another slide over it. Gently pull slides apart with an easy sliding motion to get alternate thick and thin area. (Stanley Lawrence Lamberg, 1978)
2.7.3.2 Sparsely Cellular Fluid
Clear, sparsely cellular fluid yield scanty or no sediment after centrifugation at 2000 rpm for ten minutes. Cytocentrifugation should be used for such cases. Cytocentrifuge concentrates small number of cells suspended in fluid specimens. Spinning samples at 2000 rpm for 2 minutes sediments cells directly to slides. The blotter or filter card simultaneously absorbs the fluid medium. The result is a mono layer of well preserved cells with in an area of 6 mm. major objection to the use of cytocentrifuge is the distortion of cellular morphology due to air drying artifacts, which can be avoided by immediate fixation or by using an equal volume of polyethylene glycol. The fluid is first concentrated by routine centrifugation at 2000 rpm for 10 minutes. Large portion of the supernatant is discarded leaving behind a few drops in the bottom of the centrifuge tube. This portion is sirred well and 2-5 drops (optimum 3 drops) are used for cyterifugation.(Stanley Lawrence Lamberg,1978)

2.7.3.3 Haemorrhagic Fluid:
Carnoy's fixative is used to lyse RBCs in haemorrhagic fluid. Alternatively glacial acetic acid alone or saline re-hydration technique can be used in which the smears are rapidly dried at 37 °C for 5 minutes and re-hydrated in normal saline for 30 seconds and then fixed in alcohol fixative.(Stanley Lawrence Lamberg,1978)

2.7.3.4 Cell Block Preparation:
There are different method of cellblock preparation like bacterial agar method, plasma thrombin clot method etc. An alternative method of cellblock preparation is a modified technique using AAF fixative (95% ethyl alcohol 34ml + formalin 4ml + glacial acetic acid 2ml).(Stanley Lawrence Lamberg,1978)

**Technique of cell Block preparation**
- The cell pellet remaining after preparing smears is mixed with thrice the volume of AAF fixative and one or two drops of the supernatant fluid and centrifuged foe 10 minutes at 2000 rpm.
- Re-suspend the cell button in AAF fixative and centrifuge for 10 minutes at 3000 rpm.
- Set aside the centrifuge tube for 4 – 6 hours.
2.7.4 Staining:
Pap stain or MGG is recommended for routine diagnosis. Cell loss and cell crowding is found to be very high in Pap method as compared to air-dried method. Cytoplasmic details are well preserved in Giemsa than in Pap stain. Crisp chromatin granularity is preserved in Pap stain, whereas, nuclear chromatin transparency is less in Giemsa and thus the limitation of one method can be counterbalanced in the other method.

2.8 Fixation of cytology specimens:
Rapid fixation of smears is necessary to preserve cytologic details of cells spread on a glass slide. Fixation means prevention of degeneration of cells and tissue by the autolytic enzymes present in the cells and preservation of cells as close as possible to the living state. To achieve this smears are placed in the fixative solutions for specific periods of time before the staining procedure is started. Fixation changes the physical and chemical state of the cells and determines the subsequent staining reaction that could be carried out on the smears. (George L. Wied. 1997)

Properties of cytologic fixatives
- Do not excessively shrink or swell cells.
- Do not distort or dissolve cellular components.
- Inactive enzyme and preserve nuclear details.
- Kill microbial.
- Improves of the differentiation and enhance staining properties of the tissue and cell components.

2.8.1 Cytological fixative:
2.8.1.1 Wet fixation:
A. Routine fixatives
The process of submerging of freshly prepared smears immediately in a liquid fixative is called wet fixation. This is the ideal method for fixing all gynecological and non-gynecological smears and any of the following alcohols can be used. All alcohol fixative should be discarded or filtered after each use. (George L. Wied. 1997)
1. 95% Ethyl Alcohol (Ethanol)
2. Ether alcohol mixture
3. 100% Methanol
4. 80% Propanol and Isopropanol
5. Denatured alcohol

**Time of fixation:** Minimum 15 minutes fixation prior to staining is essential. Prolonged fixation for several days or even few weeks will not affect the morphology of cells. If smears are to be preserved over a long period of time in alcohol, it is better to store them in capped container in the refrigerator.(George L.Wied. 1997)

**B. Coating fixative**

Coating fixative are substitutes for wet fixatives. They are either aerosols applied by spraying the cellular samples or a liquid base, which is dropped onto the slide. They are composed of an alcohol base, which fixes the cells and wax like substance, which forms a thin protective coating over the cells e.g. Carbowax fixative. Diaphine fixative Spray coating fixative with high alcohol content and a minimum of lanolin or oil is also an effective fixative.(George L.Wied. 1997)

**C. Special purpose fixative**

- Carnoy's fixative: this is a special purpose fixative for haemorrhagic samples. The acetic acid in the fixative haemolyses the RBCs. It is excellent nuclear fixative as well as preservative for glycogen but result in considerable shrinkage of cells and tends to produce over staining in hematoxylin. Overfixing in carnoy's also result in loss of chromatin material. Carnoy's fixative must be prepared fresh when needed and discarded after each use. It loses its effectiveness on long standing and chloroform can react with acetic acid to from hydrochloric acid.
- AAF Fixative: this is the ideal fixative used for cellblock preparation of fluid specimens.

**2.8.1.2 Mailing of unstained smears**

**Glycerine method for mailing slides:** Smears are first fixed in 95% for 12 minutes and removed. Two drops of glycerine are placed on smears and covered with a clean glass slide. This may be wrapped in wax paper and mailed to the laboratory in a
suitable container. Coating fixative such as carbowax fixative and spray coating fixative can be used primarily to facilities transport of smears, mailing etc.

2.8.1.3 Prefixation of cytologic material

Prefixation may preserve some specimens for days without deterioration of cells. Some of the disadvantages of pre-fixation are precipitation/coagulation of proteins, hardening of cells in spherical shapes and condensation of chromatin. The coagulation of proteins may interfere with the adherence of cells to glass slides. It also ‘round up’ the cells – causes the cells to gather together into tight clusters making stain absorption and interpretation difficult. Albuminized slides should be used to prepare smears from prefixed sample.(George L. Wied. 1997) The most common solutions used for this purpose are:

- Ethyl alcohol (50%).
- Sacomanno's fixative (50% alcohol with 2% Carbovax 1540).
- Mucolaxx

Many other preservatives have been developed for use with automated cytology systems.

2.8.1.4 Rehydration of Air Dried Smears

Unfixed, air-dried gynaecological smears received from peripheral areas can be used for Papnicolaou staining by rehydration method. The simplest rehydration technique is to place air dried cytological specimens in 50% aqueous solution of glycerine for three minutes followed by two rinses in 95% ethyl alcohol and then stained by the routine pap method.(George L. Wied. 1997)

2.9 Staining methods in cytology

2.9.1 Papanicolaou’s stain or PAP stain:

is a multichromatic staining cytological technique developed by George Papanikolaou, the father of cytopathology.

Pap staining is used to differentiate cells in smear preparations of various bodily secretions; the specimens can be gynecological smears (Pap smears), sputum, brushings, washings, urine, cerebrospinal fluid, abdominal fluid, pleural fluid, synovial fluid, seminal fluid, fine needle aspiration material, tumor touch samples, or other materials containing cells.(Carson, Freida L, Hladik, Christa, 2009)
Pap staining is a very reliable technique. As such, it is used for cervical cancer screening in gynecology. The entire procedure is known as Pap smear. The classic form of Pap stain involves five dyes in three solutions;(Carson, Freida L, Hladik, Christa, 2009)

- A nuclear stain, haematoxylin, is used to stain cell nuclei. The unmordanted haematein may be responsible for the yellow color imparted to glycogen.
- First OG-6 counterstain (-6 denotes the used concentration of phosphotungstic acid; other variants are OG-5 and OG-8). Orange G is used. It stains keratin. Its original role was to stain the small cells of keratinizing squamous cell carcinoma present in sputum.
- Second EA (Eosin Azure) counterstain, comprising three dyes; the number denotes the proportion of the dyes, e.g. EA-36, EA-50, EA-65.
- Eosin Y stains the superficial epithelial squamous cells, nucleoli, cilia, and red blood cells.
- Light Green SF yellowish stains the cytoplasm of other cells, including non-keratinized squamous cells. This dye is now quite expensive and difficult to obtain, therefore some manufacturers are switching to Fast Green FCF; however, it produces visually different results and is not considered satisfactory by some.
- Bismarck brown Y stains nothing and in contemporary formulations it is often omitted.

When performed properly, the stained specimen should display hues from the entire spectrum: red, orange, yellow, green, blue, and violet. The chromatin patterns are well visible, the cells from borderline lesions are easier to interpret and the photomicrographs are better. The staining results in very transparent cells, so even thicker specimens with overlapping cells can be interpreted.

On a well prepared specimen, the cell nuclei are crisp blue to black. Cells with high content of keratin are yellow, glycogen stains yellow as well. Superficial cells are orange to pink, and intermediate and parabasal cells are turquoise green to blue. Metaplastic cells often stain both green and pink at once.

Pap stain is not fully standardized; it comes in several versions, subtly differing in the exact dyes used, their ratios, and timing of the process.

The EA stain contains two mutually incompatible chemicals, Bismarck brown and phosphotungstic acid, which precipitate each other, impairing the useful life of the mixture and compromising the differential staining of eosin and light green. The descriptions of the compositions of the staining solutions vary by source and differ even in Papanicolaou's own publications. Mixtures of the same name from different
vendors therefore can differ in composition, occasionally producing different or poor results. (Carson, Freida L, Hladik, Christa, 2009)

2.9.2 **Haematoxylin&Eosin stain:**

Some laboratories use routine H&E stain for non-gynecological smears. Hematoxylin is a salt that dissociates in water into positive and negative ions. Its positive ion (basic-alkaline) readily combines with negatively charged regions of cellular macromolecules, especially phosphate groups of nucleic acids, coloring them blue to purple to black.

Any substance, cell or tissue, that tends to be stained in this way by hematoxylin is said to be basophilic. Because of their high nucleic acid (DNA and RNA) content, the nuclei of cells are usually basophilic. However, the cytoplasm of cells is generally not basophilic unless it contains unusually high amounts of RNA (or another macromolecule with appropriate negative charge regions).

Eosin is also a salt that dissociates in water into ions. Its negative ion, which is acidic in nature, readily combines with positively charged regions of cellular macromolecules, especially positively charged regions of cytoplasmic proteins, coloring them a variety of hues, ranging from pink to red to orange.

Any substance, cell or tissue to be stained in this way by eosin is said to be acidophilic or eosinophilic. With H&E staining, therefore the nuclei of the cells of virtually all tissues stain bluish and the cytoplasmic portions of the cells of most tissues stain pink. (Claude gompel, John Wiley, Sons 1978)

2.9.3 **Diff-Quik staining:**

is a commercial Romanowsky stain variant, commonly used in histological staining to rapidly stain and differentiate a variety of smears, commonly blood and non-gynecological smears, including those of fine needle aspirates. It is based on a modification of the Wright Giemsa stain pioneered by Bernard Witlin in 1970. It has advantages over the older Wright Giemsa staining technique, as it reduces the 4 minute process into a simplified 15 second operation, and allows for selective increased eosinophilic or basophilic staining depending upon the time the smear is left in the staining solutions.
Diff-Quik is utilized on material which is air-dried prior to alcohol fixation. The primary use of Romanowsky-type stains is for cytoplasmic detail, such as intracytoplasmic mucins, fat droplets and neurosecretory granules. Extracellular substances, such as free mucin, colloid, ground substance, etc., are also easily stained, and appear metachromatic. Microbiologic agents, such as bacteria and fungi, also appear easier in Diff-Quik. (Demay, November 2006)

The Diff-Quik stain consists of 3 solutions:
- Diff-Quik fixative reagent
  - Triarylmethane dye
  - Methanol
- Diff-Quik solution I (eosinophilic)
  - Xanthene dye
  - pH buffer
  - Sodium azide
- Diff-Quik solution II (basophilic)
  - Thiazine dye
  - pH buffer

2.10 The next diagnostic step after cytology:
The next diagnostic step after cytology is histology. Histology is the microscopic examination of samples of whole tissue, and it is performed on a solid piece of tissue that has been collected surgically from the pet. Histology focuses on tissue architecture, and provides information about how cells are organized within a tissue, and how tissues interact with each other.

In most cases, histology will provide a definitive diagnosis, and is generally considered the diagnostic "gold standard". Histology is often needed to determine if a tumor is benign or malignant, and is routinely recommended to confirm the cytological findings. If your pet has a growth surgically removed, always request that the tissue be sent away for histological examination. (Kristiina Ruotsalo, December 2, 2008)
3.1 Study design and duration:
Descriptive cross-sectional study aimed to compare between different cytology stains during the period from April 2017 to July 2017.

3.2 Study area:
Medical Laboratory in department of Histopathology and cytology, Faculty of Medicine, University of Gezira.

3.3 Study populations:
This study was done in different cytology samples.

3.4 Sample size:
Hundred twenty cytology samples were collected and dividing according to cytology samples types.

3.5 The Method:
Hundred twenty samples were collected according to the type of sampling procedure, smear preparation, fixation (95% ethyl alcohol or in other substitutes for a minimum of 15 minutes and the smears of Diff-Quik stain fixation air rapid dry) and staining with different stains (Pap, Diff-Quik and H&E stain), taking into account quality control.

3.5.1 Cytological staining:
3.5.1.1 Protocol of Papnicolaou stain:
Ethyl alcohol fixed smear are hydrated in 95% alcohol for 2 min, through 70% alcohol for 2 min, rinse in water for 1 min, stained in harries hematoxylin for 5 min, rinsed in water for 2 min, differentiated in 0.5% aqueous hydrochloric acid for 10 seconds, rinsed in water for 2 min, blued in Scott's tap water substitute for 2 min, rinsed in water for 2 min, dehydrated in 70% alcohol for 2 min, dehydrated in 95% alcohol for 2 min, dehydrated in 95% alcohol for 2 min, stained in OG6 for 2 min, rinsed in 2 changed 95% alcohol for 2 min in each, stained in EA50 for 3 min, dehydrated in 95% alcohol for 1 min, through absolute alcohol, cleared in xylene and mounted in DPX (John D Bancroft and Marilyn Gamble, 2006)
- Nuclei – blue
- Cytoplasm of superficial cells – pink
- Cytoplasm of intermediate cells, parabasal cells – Blue Green
- Red blood cells – bright red
- White cells – pale blue cytoplasm

3.5.1.2 Protocol of Diff-Quik stain:
slide air dry, fix in "Diff Quik" fixative for 30 secs, place in "Diff Quik" solution II for 30 secs, counterstain "optional" with "Diff Quik" solution I for 30 secs, rinse in tap water to remove excess stain for 10 secs, rapidly dehydrate in absolute alcohol and clear and mount. (John D Bancroft and Marilyn Gamble, 2006)
- Nuclei – blue
- Cytoplasm of superficial cells – pink
- Erythrocytes – Pink/yellowish red

3.5.1.2.1 Protocol of Haemotoxylin&Eosin stain:
Slide are hydrate through graded alcohol to water every change 2min, Stain in haematoxyline for 6 min, wash in tape water for 10 sec, differentiate in 1% acid alcohol for 10 sec, wash well in raining tape water until smears blue for 5min, stain in eosin for 2min, wash in raining tap water for 2 min and dehydrate through alcohol ,clear and mount. (John D Bancroft and Marilyn Gamble, 2006)
- Nuclei – blue/black
- Cytoplasm – varying shades of pink
- Red blood cells – orange red
- Fibrin – deep pink

3.5.2. Sample analysis:

3.5.2.1. Assessment of staining smears and staining quality:
The smears were assessed and evaluated according to quality of stains and the appearance of the nucleus and cytoplasm and each slide was given a degree according to quality of the staining results.

3.6 Data analysis:
The Data was analyzed by frequency.
3.7 Ethical considerations:

- The samples collected under privacy and confidentiality.
- The samples not used for any other purpose.
- Also there are no side effects after taking the sample.

3.8 Materials:

- All materials used in this study were mentioned in appendix.
Chapter Four
Result and Discussion

4.1 Result

Table (4-1) Appearance of nucleus, cytoplasm and general cytological appearance in samples according to Papanicolauo stain.

<table>
<thead>
<tr>
<th>Stain</th>
<th>FNA</th>
<th>Sputum</th>
<th>Urine</th>
<th>Buccal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excellent</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Very Good</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Good</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table (4-2) Appearance of nucleus, cytoplasm and general cytological appearance in samples according to Diff-Quik stain.

<table>
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<th>FNA</th>
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<th>Urine</th>
<th>Buccal</th>
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</thead>
<tbody>
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<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Very Good</td>
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<td>9</td>
</tr>
<tr>
<td>Good</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

Table (4-3) Appearance of nucleus, cytoplasm and general cytological appearance in samples according to Haemotoxylin&Eosin stain.

<table>
<thead>
<tr>
<th>Stain</th>
<th>FNA</th>
<th>Sputum</th>
<th>Urine</th>
<th>Buccal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excellent</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Very Good</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>2</td>
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<tr>
<td>Good</td>
<td>6</td>
<td>9</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>
Figure(4-1) Photo microscopic showing sputum smear stained by Papanicoluao stain.

Figure(4-2) Photo microscopic showing sputum smear stained by Diff-Quik stain.

Figure(4-3) Photo microscopic showing sputum smear stained by Haemotoxylin&Eosin stain.
Figure(4-4) Photo microscopic showing buccal smear stained by Papanicoluao stain.

Figure(4-5) Photo microscopic showing buccal smear stained by Diff-Quik stain.

Figure(4-6) Photo microscopic showing buccal smear stained by Haemotoxylin&Eosin stain.
Figure(4-7) Photo microscopic showing FNA smear stained by Papanicolaou stain.

Figure(4-8) Photo microscopic showing FNA smear stained by Diff-Quik stain.

Figure(4-9) Photo microscopic showing FNA smear stained by Haemotoxylin&Eosin stain.
Figure (4-10) Photo microscopic showing urine smear stained by Papnicolaou stain.

Figure (4-11) Photo microscopic showing urine smear stained by Diff-Quik stain.

Figure (4-12) Photo microscopic showing urine smear stained by Haemotoxylin&Eosin stain.
4.2. Discussion

The choice and select of an appropriate stain for cytology smears consider the basic entry to obtain reliable and adequate information result. Furthermore decrease the rates of false negative.

According to this study finding Papnicolaou stained slides in FNA smear were excellent in nuclear details, cytoplasm and general cytological appearance, this is agree with study done by (Shehnaz Khan, 2012) and disagree with study done by (Dr. S. Suryalakshmi, 2016) finding Haemotoxylin and Eosin stain is excellent in nuclear details, cytoplasm and general appearance.

The study reveal that Papnicolaou stained slides in sputum sample were excellent in nuclear details, cytoplasm and general cytological appearance, our finding is agree with study done by (A. S. Ammanagi, V. D. Dombale and S. S. Sangolli) and disagree (Jessica Goncalves) finding Diff-Quik stain is excellent in nuclear details, cytoplasm and general appearance.

Our finding indicate that Papnicolaou stained slides in buccal smear were excellent in nuclear details, cytoplasm and general cytological appearance, this is agree with study done by (Sohair B A Ayyad, 2006).

According to this research finding Papnicolaou stained slides in urine sample were excellent in nuclear details, cytoplasm and general cytological appearance, this is agree with study done by (Luis Alberto Palaoro).

According to this study Papnicolaou stain was the best stain but takes more time and expensive followed by Diff-Quik stain takes short time and cheap cost and Haemotoxylin and Eosin stain takes moderate time and moderate cost.
Chapter Five

Conclusion and recommendation

5-1 Conclusion:

The study found that the best stain for the demonstration of urine, sputum, buccal and Fine Needle Aspiration samples is Papanicolaou stain followed by Diff-Quik stain and Hematoxyline and Eosin stain.

5-2 Recommendations:

This study recommended the use of Papanicolaou stain as routine stain for cytological smears.
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• website: http://otago.ac.nz/

• website: http://otago.ac.nz/
Appendices

Appendix I (Materials)

Materials:

- Lab and lab coat
- Gloves
- Filter papers
- Diff-Quik stain
- Papnicolaou stain
- Haemotoxylin&Eosin stain
- Microscope
- Pencil
- Containers
- Xylene.
- D.P.X.
- Coplin jar.
- Swab.
- Three slides boxes and three cover glass box.
Appendix II (solution)

- **Harris’ hematoxylin**:  
  Hematoxylin = 5g  
  Ethanol = 50ml  
  Potassium alum = 100g  
  Distilled water (50°C) = 1000ml  
  Mercuric oxide = 2-5g  
  Glacial acetic acid = 40ml

- **Orange G 6**:  
  Orange G (10% aqueous) = 50ml  
  Alcohol = 950ml  
  Phosphotungstic acid = 0-15g

- **EA 50**:  
  0.04 M light green SF = 10ml  
  0.3M eosin Y = 20ml  
  Phosphotungstic acid = 2g  
  Alcohol = 750ml  
  Methanol = 250ml  
  Glacial acetic acid = 20ml

- **Fixative solution**:  
  1.8 mg/L Triarylmethane dye in methyl alcohol.

- **Diff-Quik Solution I**:  
  1 gm/L Xanthene Dye, buffer and sodium azide (0.01% as preservative).

- **Diff-Quik Solution II**:  
  1.25 gm/L Thiazine Dye Mixture and buffer

- **Mayer's Haematoxylin (100ml)**:  
  - Hematoxylin 1 g  
  - Potassium alum 25 g  
  - citric acid 0.5 g  
  - Sodium iodate 0.1 g  
  - Chloral hydrate 25 g
- D.W 500 ml

**Eosin (50ml):**
- Eosin 5 g
- D.W 50 ml