Biomedical Research and Therapy 2016, 3(2): 490-500
ISSN 2198-4093
www.bmrat.org

DOI 10.7603/s40730-016-0006-8

REVIEW

Evolution of Pap Stain

Kalyani Raju

Professor of Pathology, Sri Devaraj Urs Medical College, Sri Devaraj Academy of Higher Education and Research, Kolar. Karnataka. India
*Corresponding author: drkalyanir@rediffmail.com

Received: 31 Jan 2015 / **Accepted**: 12 Feb 2016 / **Published online**: 25 Feb 2016

Abstract— Pap stain was first developed by Dr GN Papanicoloau in 1947 and since then it has been used successfully to screen cervical cancer. In fact, it has reduced the incidence of cervical cancer by 70% especially in developed countries having well planned screening programs. However, the stain has undergone various modifications from regressive conventional method to progressive rapid Pap staining where the time taken for staining was reduced. Further the stain was modified as ultrafast Papanocoloau stain and modified ultrafast Pap stain were air dried cervical smears were used and staining time was reduced. Later the other modifications were Enviro-Pap stain which was environmentally friendly with results similar to conventional method; REAP stain which was rapid and economical and Cytocolor developed by Merck where isopropyl alcohol is replaced by Propanol. Each method has advantages and disadvantages. Hence laboratories should develop their own protocol and standardize the staining technique.

Keywords— Cervical cancer screening, conventional Pap stain, Cytocolor, Enviro-Pap stain, Pap stain, Rapid Pap stain, REAP stain..

INTRODUCTION

Pap stain is a universal stain used for gynecologic and non-gynecologic cytology smear. It is mainly used for oral and cervical cancer screening in asymptomatic population and in the follow up of patients with cancer. Pap test has decreased incidence of cervical cancer by 70% in developed countries. Pap stain yields polychromatic transparent staining reaction with crisp nuclear and cytoplasmic features (Asthana A, 2014; RoyBiswas et al., 2008).

Pap stain was developed by Greek doctor George Nicholas Papanicoloau at Cornell medical college of New York to know the variation in cellular maturity and metabolic activity in vaginal smears. The original pap stain was published in 1942 and later it was modified by Dr. Papanicoloau in 1954 and 1960 (Asthana A, 2014; Bales, 2006; GN, 1960; Papanicolaou, 1942, 1954). Since the introduction of pap stain by G.N. Papanicoloau it has undergone various modifications (Asthana A, 2014).

The principle of pap stain

The principle of pap stain is to clearly distinguish between basophilic and acidophilic cell components and obtain a detailed chromatin pattern. The pap stain has 3 solutions having 6 dyes. Solution 1 has Hematoxylin stain, a basic nuclear stain. Solution 2 has OG-6, an acidic cytoplasmic stain, has orange G with phosphotungstic acid (6 denotes the used concentration of phosphotungstic acid). Orange G is an acid dye and stains keratin with a bright intense orange. Phosphotungstic acid, a mordant, strongly binds to protein and helps to intensify the color achieved. Solution 3 has EA (PH 4.5-5 for maximum results) which is a polychromatic stain having 3 components; light green/fast green, eosin Y and Bismarck brown Y. Light green SF is an acid dye which stains the cytoplasm of metabolically active

cells i.e. intermediate squamous cells, parabasal cells, endocervical cells, histiocytes, leukocytes, undifferentiated carcinoma cells and cells from adenocarcinoma as green. Eosin Y is an acid dye which stains cytoplasm of superficial squamous cells, nucleoli, erythrocytes and cilia. Bismarck brown Y precipitates phosphotungstic acid, the ingredients responsible for differential staining by light green and eosin. Fast green sometimes used in modified EA which has a lower solubility in alcohol and makes it less prone to being washed away from cells. Fast green is not as stable as light green. EA 36/50 has 2 times more light green than EA65 and hence former is preferred for gynecologic smears and latter for non-gynecologic smears/thick smears. However EA65 differentiates adenocarcinoma of endocervix which takes pink stain from that of endometrium which takes blue stain. A well stained smear shows well stained nuclear chromatin with differential cytoplasmic counterstaining and cytoplasmic transparency (Bales, 2006; Catherine M Keebler, 2008,). The greenish cytoplasmic stain of neutrophils are taken as internal control.

Pap staining procedure

Pap staining procedure is classified into two types i.e progressive and regressive method. In progressive method the nucleus is stained to the intensity required followed by bluing agent. Mayer Hematoxylin and Gill's hematoxylin is preferred. In regressive method the smear is over stained with nuclear stain with a non-acidified hematoxylin. The excess stain is removed with dilute HCL acid solution. Harris hematoxylin is preferred for regressive method. The progressive method is the most commonly used method in laboratories today. It is easier to control and provides more stability on a day to day basis and also for automation (Catherine M Keebler, 2008,). Conventional staining was time consuming, hence Rapid pap staining was developed.

Conventional pap staining procedure

It takes 20-30 minutes. Fixation is made with 95% ethanol / ether and ethanol. The steps are shown in Figure 1. Harris hematoxylin without acetic acid is used (Catherine M Keebler, 2008,). The conventional Pap stain has 24 solutions, of which three are acid dyes, six water, one is chemically defined bluing agent, eight 95% ethanol, three absolute alcohol and three xylene(Gill, 2006). The cytomorphology with conventional Pap stain is shown in **Figure 2**.

Rapid Pap staining procedure

It was developed by different scientists as Kline, Tao and Sato which took 4 minutes, 5 minutes and 90 seconds respectively with fixation time of 1-2 minutes (Asthana A, 2014; Papanicolaou, 1954). The procedure is (John Hopkins cytopathology laboratory, Baltimore) is shown in **Figure 3** (Catherine M Keebler, 2008,). The cytomorphology with rapid Pap stain is shown in **Figure 4 & 5**.

The conventional and rapid Pap staining methods had many disadvantages; wet fixation was time consuming, show drying artifacts especially at edges of the smear which may lost for interpretation, hemorrhagic background, less retention of material (cell loss), smaller/discoid appearance of cells, elaborated/time consuming, requires substantial volume of ethanol (is a dehydrating agent) which is expensive, need license and renewal. In addition, the concentration of supplied alcohol is sometimes questionable and the quota of supply is limited and irregular. The colour preservation is not long lasting (Asthana A, 2014; Izhar et al., 2014; Kamal et al., 2000; RoyBiswas et al., 2008). Pap stain is expensive. In addition alcohol and xylene are inflammable solutions and has to be stored safely. Xylene is costly to dispose, in fact more expensive to dispose than purchase. The quality of rapid Pap staining was not satisfactory with suboptimal cell morphology (Gill, 2006).

In the process to overcome the disadvantages, air dried smear staining with MGG, Diff quick, Toluidine blue was considered which have less turnaround time and cell size was magnified. However, morphology of cells was not crisp with subtle nuclear features, opacity of nuclei and flatness of image in air dried smears (Asthana A, 2014; Kamal et al., 2000).

Hence for modification of Pap stain the criteria considered wereto decrease time of turnaround; cheaper/cost effective; to improve the staining quality giving unequivocal cells morphology, to find alternative method of alcohol cost effective method and stain can be used for FNAC smear (Choudhary et al., 2012; Izhar et al., 2014; Kamal et al., 2000; RoyBiswas et al., 2008).

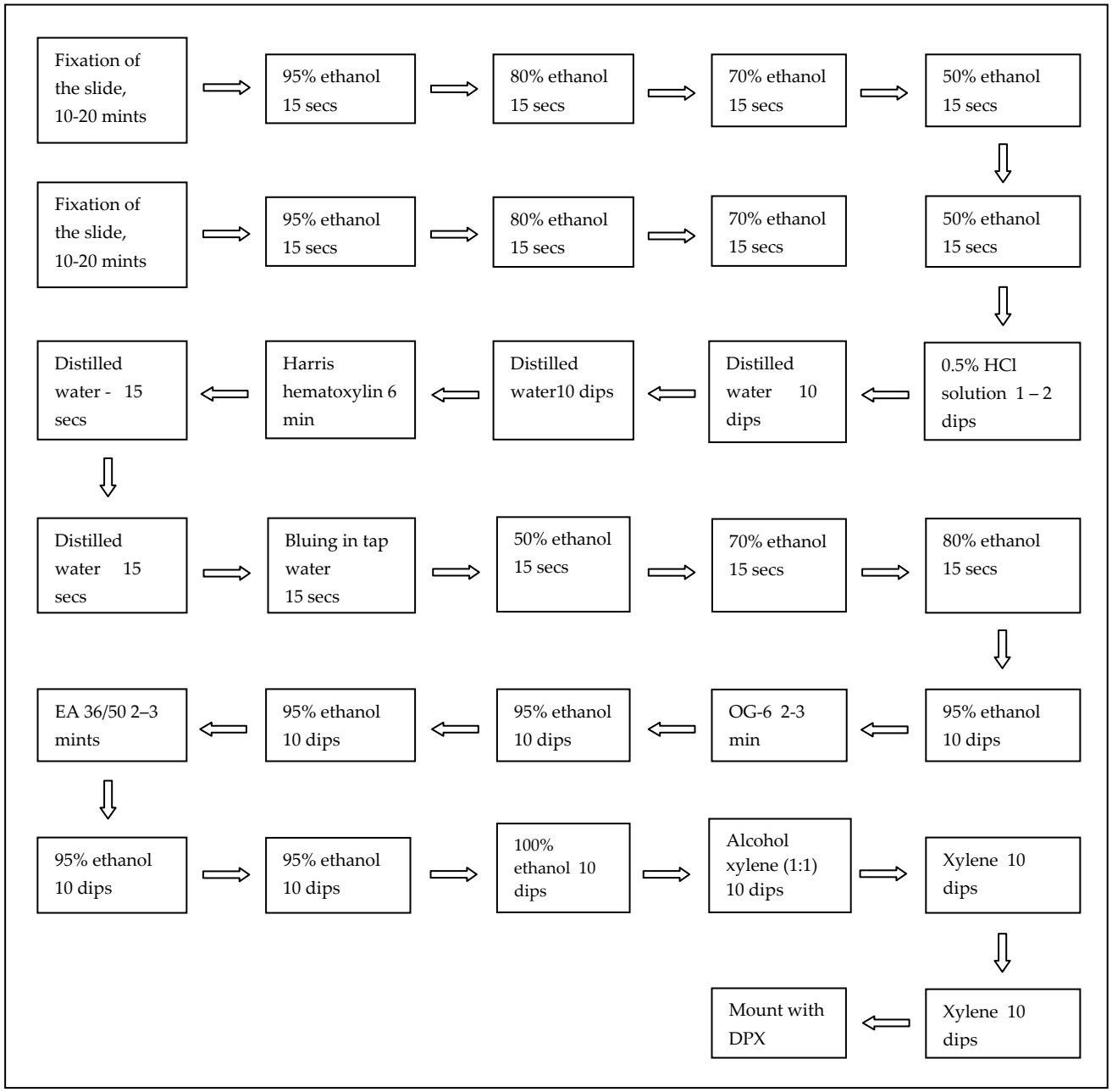

Figure 1. Conventional Pap staining procedure.

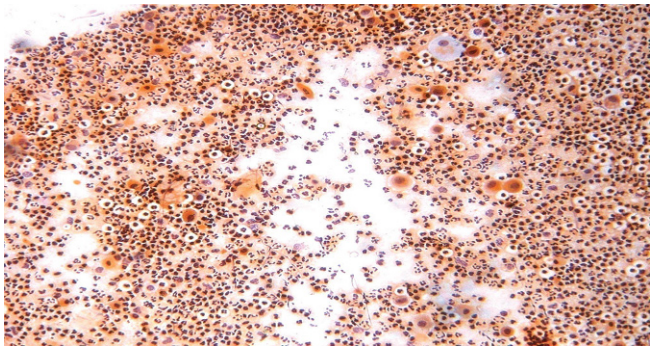

Figure 2. Shows the microphotograph of Conventional Pap stain. (X400)(Asthana A, 2014)

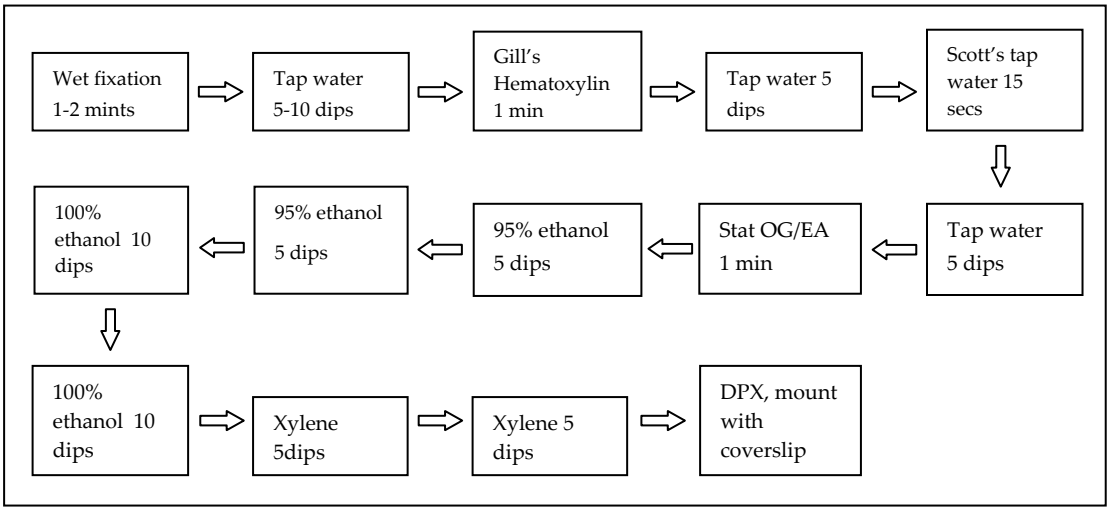

Figure 3. Rapid Papanicoloua staining technique.

Ultrafast Papanicolaou stain (UFP)

It is a modified Pap stain, first introduced by Young and Alvarez in 1995 (Yang and Alvarez, 1994). It is a hybrid of Romanowsky and conventional pap stain with turnaround time of 90 sec (Choudhary et al., 2012; Kamal et al., 2000; Yang et al., 2001). The principle is rehydration of air dried smear with normal saline followed by fixation in alcoholic formalin and Pap stain with each step duration is shortened. Alcoholic formalin consists of 300 ml of 40% formaldehyde, 2053 ml of 95% ethanol and 647 ml of distilled water. The procedure is shown in **Figure 6** (Bales, 2006). The cytomorphology with Ultrafast Pap stain is shown in **Figure 7**.

Richard Allan cytostain consists of alcoholic mixture of orange G, eosin Y, light green and aniline blue (Kamal et al., 2000). Cells are air dried and hence looks larger, flatter, more transparent than wet fixed cells with increased resolution which is required for FNAC smears. Rehydration of smears causes lysis of RBCs giving rise to blood free clear background that unmasks the cellular material for morphological evaluation. Fixation in alcoholic formation sets the condition for the differentiation of RNA from DNA in subsequent staining because of acidic PH (5) of alcoholic formation giving rise to red color nucleoli and vibrant colors of the cells (Choudhary et al., 2012; Kamal et al., 2000).

The advantages of UFP is; it gives transparent polychromatophilic stain of cytoplasm, crisp nuclear details, clear background, excellent cytologic details especially for malignant lymphoma and thyroid tumor smears. In addition, it avoids cell loss as in wet fixation due to rapid plugging of slides in fixatives / during transport, smears are prepared with ease with no physical hurry for immediate wet fixations, air drying of smears causes cells to stick firmly / flat on glass slide and not in 3 dimensional clusters. It is also preferred for intraoperative cytology (Choudhary et al., 2012; Kamal et al., 2000).

The disadvantages are; the reagents Richard Allan hematoxylin and Richard Allan cytostain are not available universally. 95% ethyl alcohol is expensive. In addition, OG component may be redundant if smears have negligible cytoplasmic keratin. Some thyroid aspirate smears shared nuclear ground glass appearance as an artifact. Universal standardization of staining solutions of UFP stain is a mandatory requisite (Choudhary et al., 2012; Kamal et al., 2000).

Modified Ultra fast Pap by Gill (MUFP)

It is a modified stain of UFP where Richard Allan haematoxylin, Richard Allan cytostain and ethyl alcohol are replaced by Gill's hematoxylin, modified EA (consisting of alcoholic mixture of Eosin Y light green, phosphotungstic acid and glacial acetic acid without orange G) and isopropyl alcohol respectively. It takes 130 sec(Choudhary et al., 2012). The procedure is shown in **Figure 8** (Choudhary et al., 2012). The cytomorphology with modified ultrafast Pap stain is shown in **Figure 4 & 5**.

MUFP takes 40 seconds extra than UFP because 30 secs in Gill's hematoxylin jar instead of 2 slow dips in Richard Allan Hematoxylin and 15 secs in modified EA jar instead of 4 sec (slow dips) in the cytostain jar of UFP. OG was omitted because of nuclear, cytoplasm and background orange discoloration (Kamal et al., 2000). The advantages of MUFP are; fast, reliable, staining time is 130 secs, no wet fixation, no poor fixation / drying artifacts as in wet fixation, cell loss as in wet fixation avoided, clear background, cytomorphology well appreciated (increased quality index), cells appear large with crisp nuclear/cytoplasmic details, nuclei open/vitreous, no distortion of nuclear membrane, nucleoli distinct & stain red in color, enhances features like anisonucleosis, chromatin / chromocenter stain well, chromatin granules dispersed/sharp, cytoplasm light green to blue & transparent, no deleterious effects on immunophenotyping, recommended for lipid rich tumors like lipoma, more useful for FNAC smears especially for diagnosis of thyroid aspirates, RS cells of Hodgkin's lymphoma, smears with negligible cytoplasmic keratinization and helps in rapid assessment of adequacy of samples with rapid diagnosis especially in intraoperative cytological diagnosis. The staining solution can be prepared from locally available reagents and hence useful in developing countries like India (Choudhary et al., 2012; Kamal et al., 2000).

The disadvantages are; because of omission of orange–G, appreciation of cytoplasmic keratinization is difficult. The other disadvantages are inadequate drying gives suboptimal results (technique sensitive), thick smears do not give satisfactory results, normal saline should be changed regularly, bipolar single nuclei not stained properly, locally available solution may influence the results adversely so requires universal standardization, the solutions is storage sensitive and the pH of the alcoholic formalin should be maintained at 5.0 otherwise can lead to poor staining (Choudhary et al., 2012).

Some authors are of opinion that MUFP cannot replace standard pap stains for final interpretation of smears (Kamal et al., 2000). Kamal from India further modified MUFP stain by replacing Gill's hematoxylin to Harris hematoxylin which is readily available in India and it does not alter the staining characteristics giving equally good results (Choudhary et al., 2012).

Enviro-Pap

It is an environmentally friendly, inexpensive, alternative Pap stain developed in 1995; the results are comparable to conventional Pap stain with respect to stain colour, optical density and distribution within the cells. The procedure follows the same sequence as conventional Pap staining (Bales, 2006; Gill, 2006). The staining procedure is shown in Figure 9 and the cytomorphology in Figure 10. In staining technique, two changes of tap water remove carbowax, Gill's Hematoxylin colours chromatin red, two changes of tap water remove excess hematoxylin, tap water for two minutes blues red hematein, tap water removes residual dye, OG-6 colours keratin yellow, three changes of 0.5% acetic acid rinse and keep OG in cells,

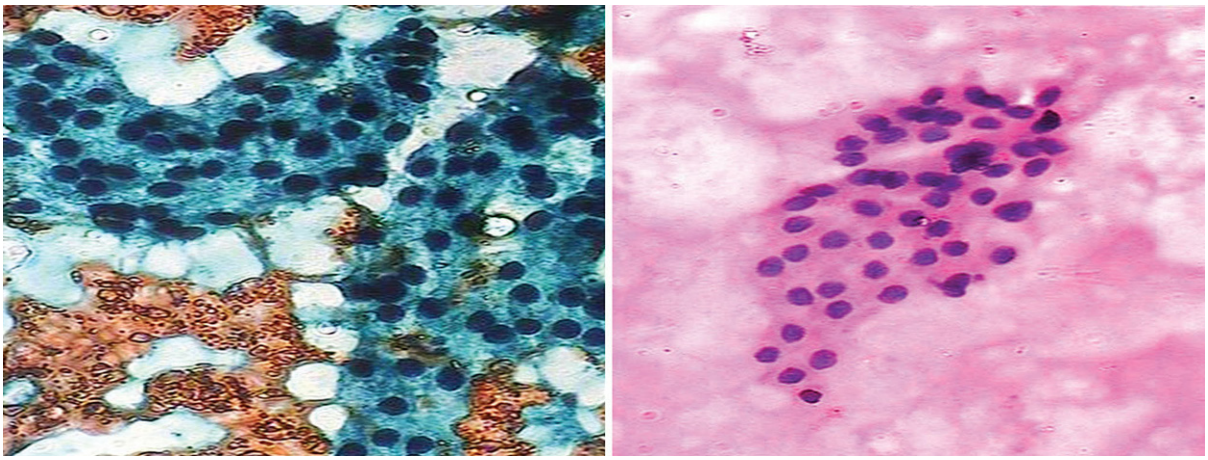

Figure 4. Shows comparison of Rapid Pap (left) with Modified Ultrafast Pap stain (right) in thyroid aspirate. (X100)(Choudhary et al., 2012)

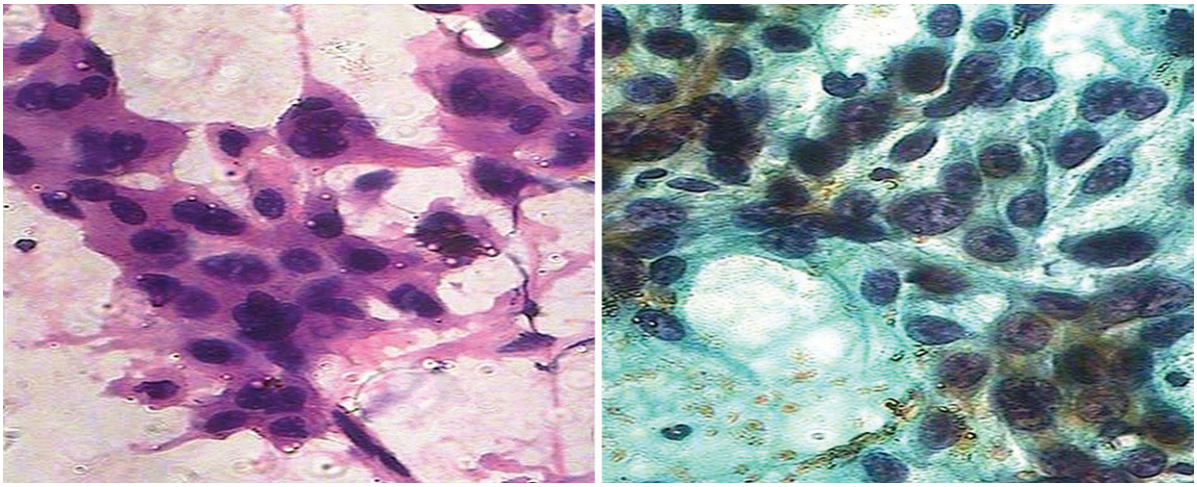

Figure 5. Comparison of Rapid Pap (left) with Modified Ultrafast Pap stain (right) in metastatic squamous cells.(X400) (Choudhary et al., 2012)

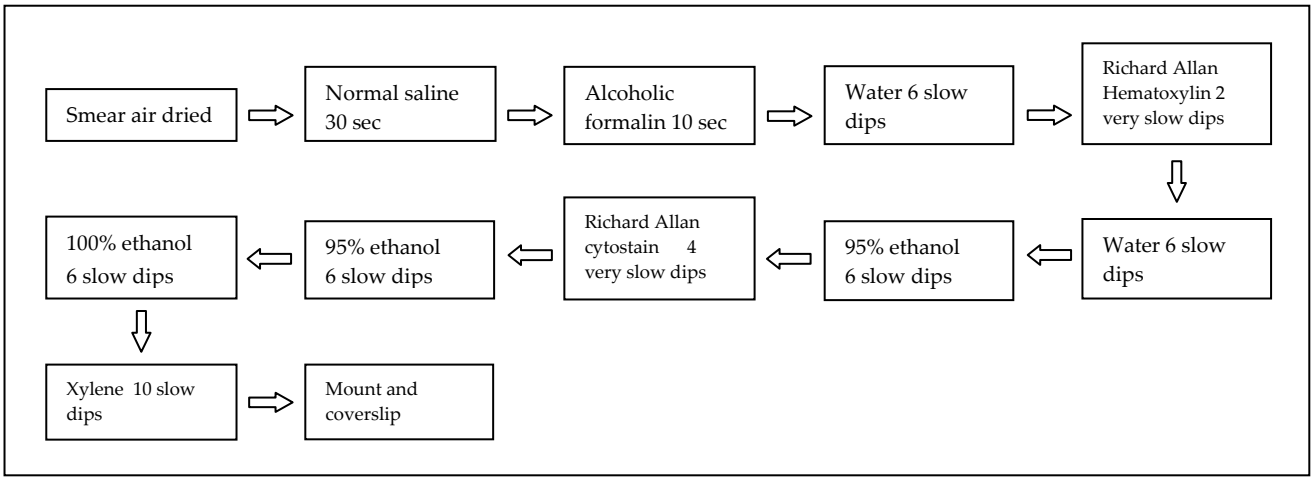

Figure 6: Ultrafast Papanicoloau staining procedure.

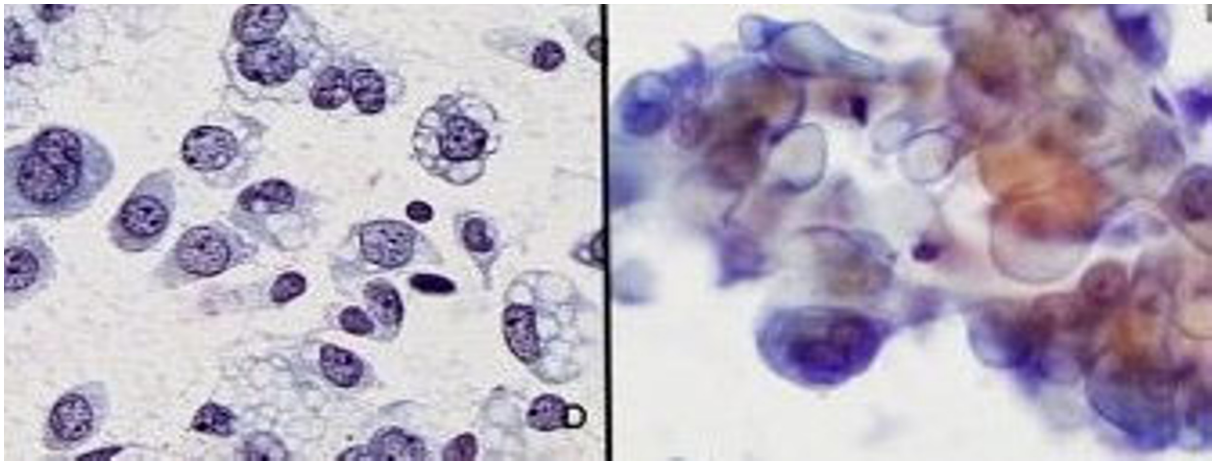

Figure 7: Comparison between Ultrafast Pap stain(left) with conventional Pap stain (right) in thyroid aspirate (X400) (Yang and Alvarez, 1994)

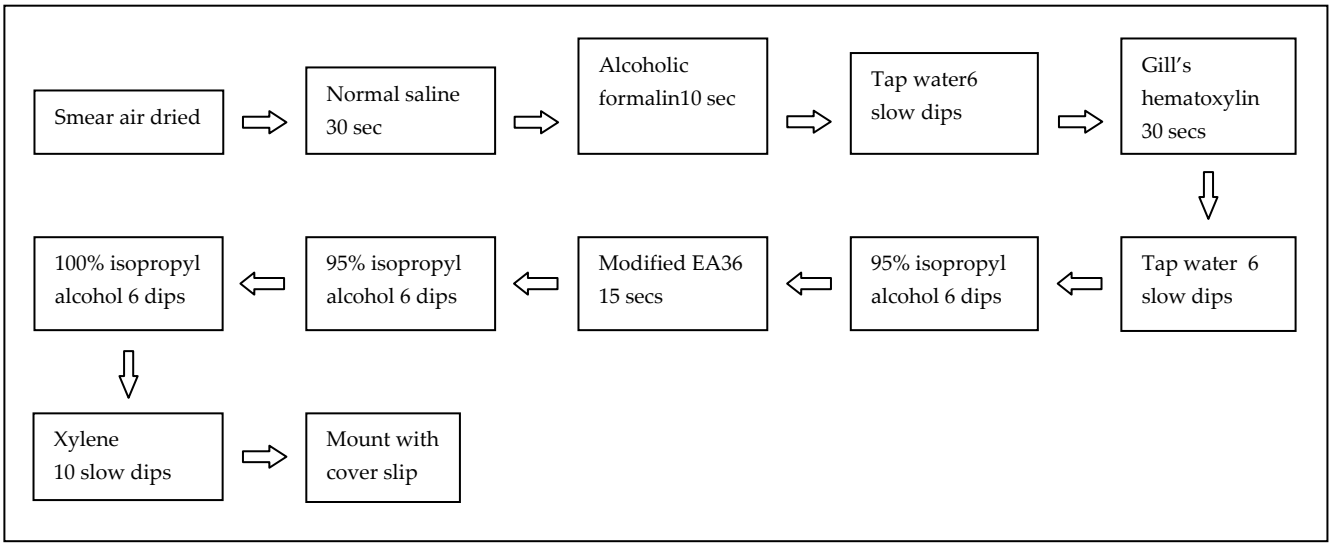

Figure 8. the Modified Ultrafast Papanicoloau staining procedure.

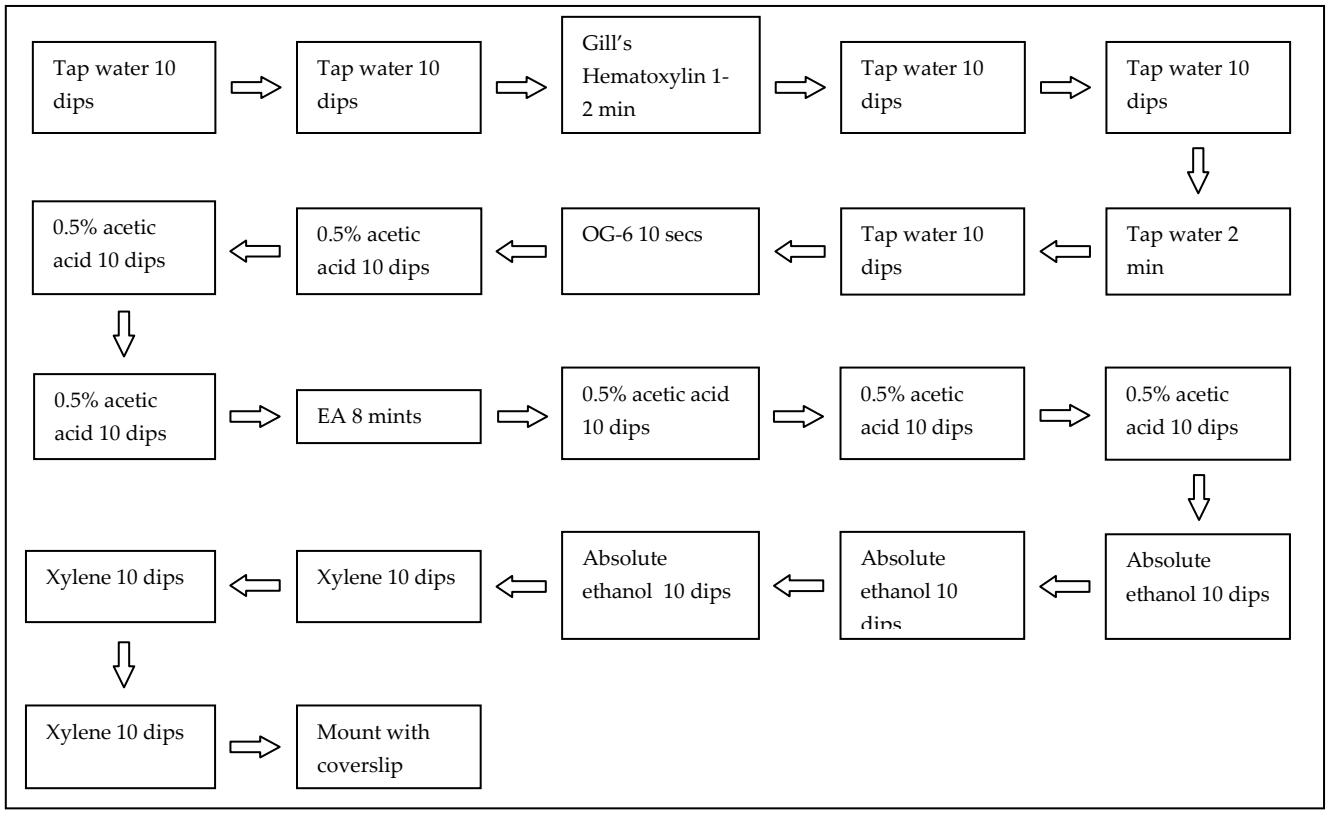

Figure 9. Staining procedure of Enviro-Pap.

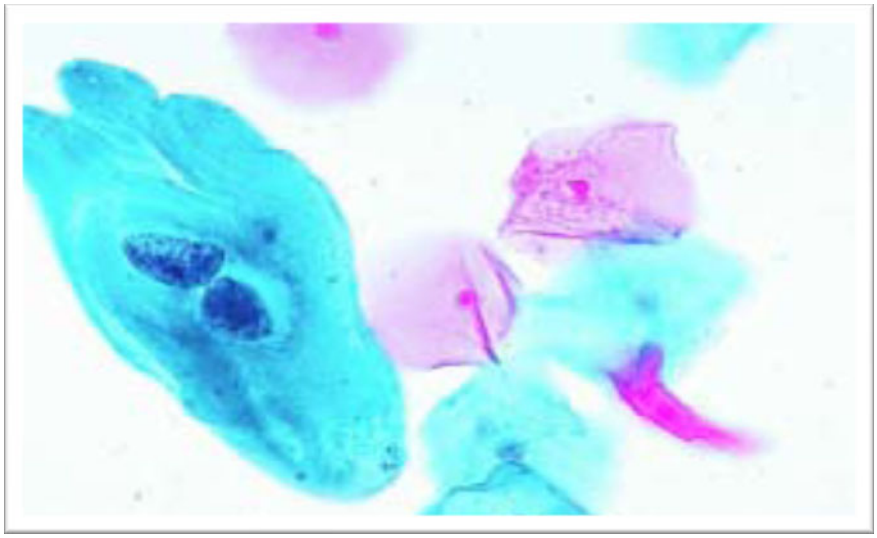

Figure 10. Cytomorphology with Enviro-Pap stain (X400) (Gill, 2006)

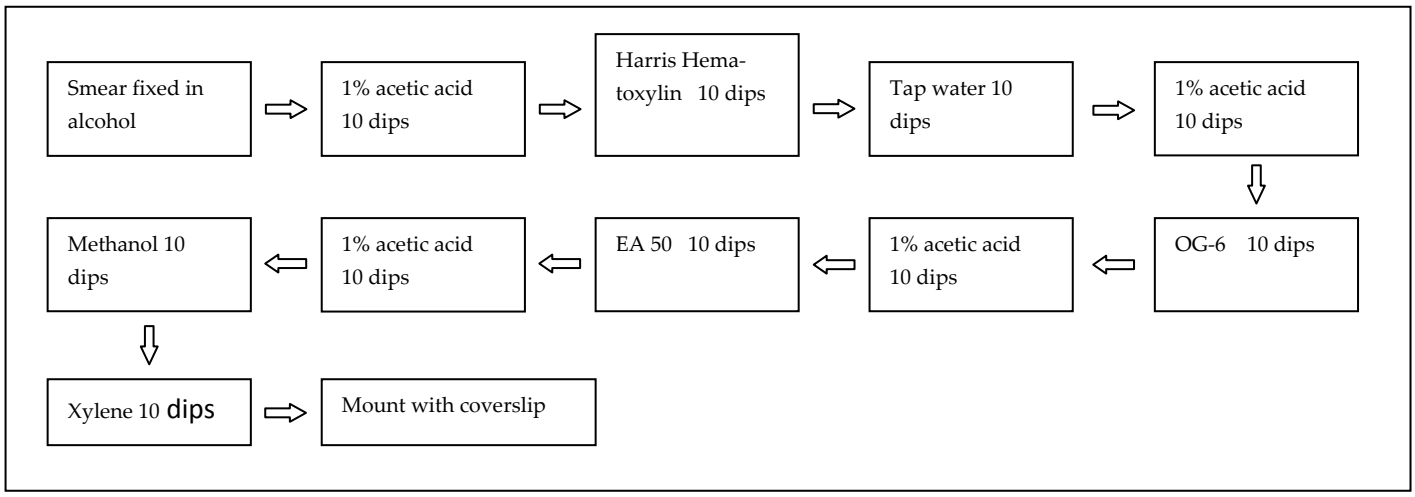

Figure 11. Staining procedure of REAP.

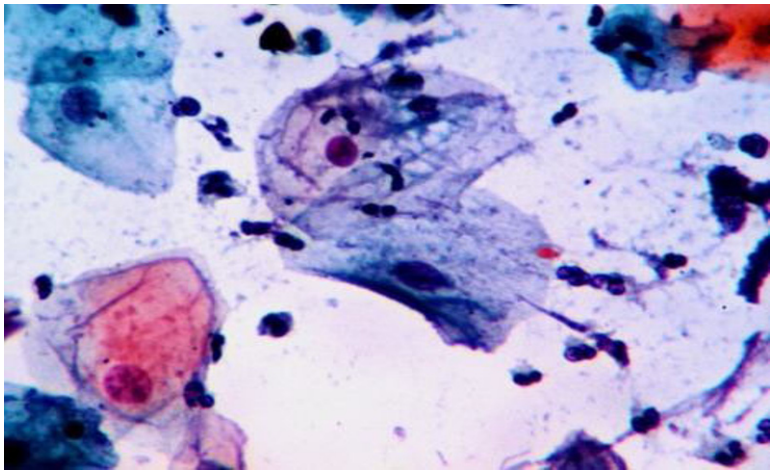

Figure 12. Cytomorphology with REAP stain (X400). (Asthana A, 2014)

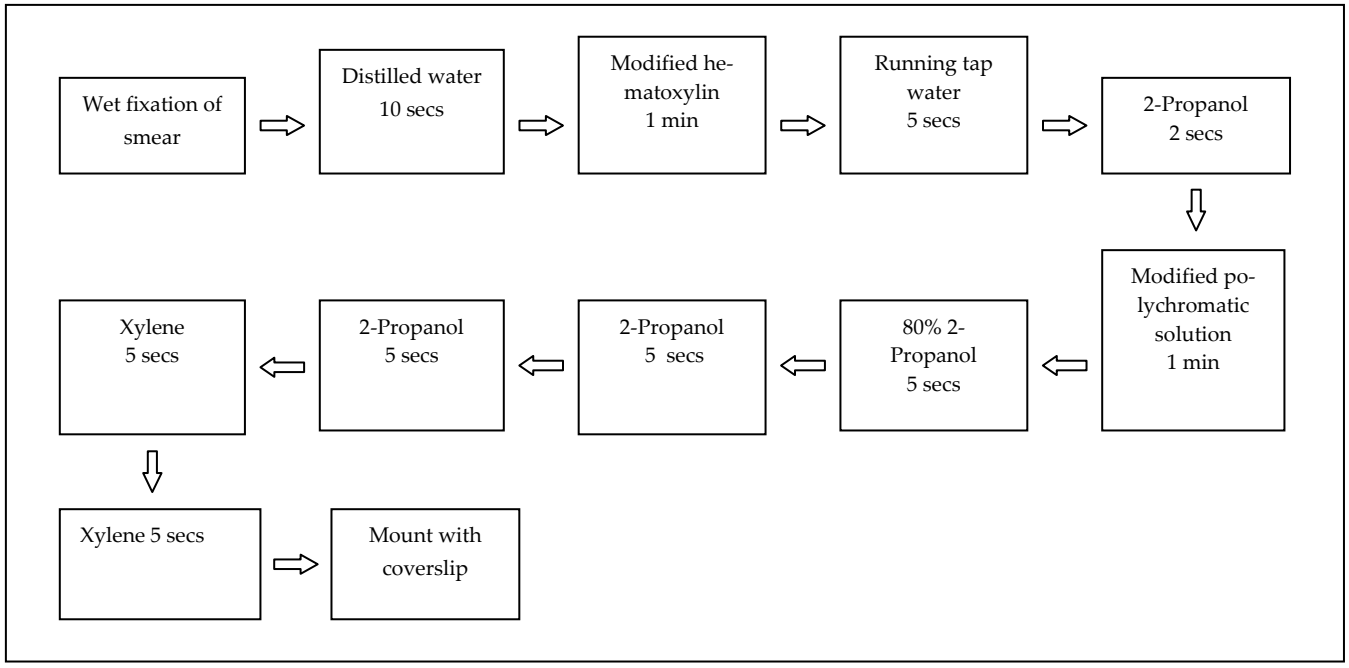

Figure 13. Staining procedure of Cytocolor.

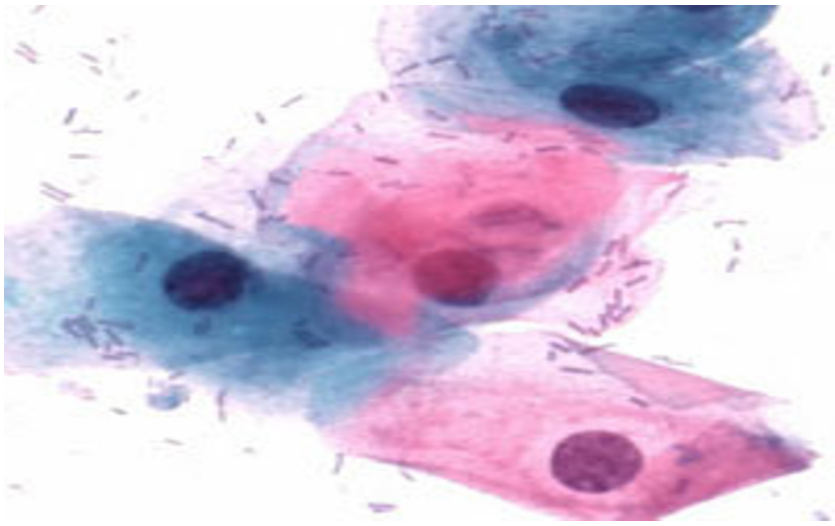

Figure 14. Cytomorphology with Cytocolor (X400) (www.merkmilipore.com/IN/en/producy/CYTOCOLOR)

EA stains cytoplasm pink-red / green, three changes of 0.5% acetic acid rinse and keep EA in cells, three changes of absolute ethanol for dehydration, three changes of xylene for clearing. Buccal smear is simultaneously stained and used as quality assurance (Gill, 2006).

But some changes are done in Enviro-Pap are:

1. The carbowax of spray fixative is removed by water instead of 95% alcohol and cross checking with microscope using crossed polarizing filters.
2. No graded alcohol used.

3. Bluing is done by tap water instead of chemical bluing (Scott's bluing agent). Bluing is best done with tap water pH of 5-7. However, variation of pH does not affect bluing and it is confirmed by bluish tinge of smear by naked eye and by microscope. It takes not more than 2 minutes.
4. Pre-OG alcohol is replaced with tap water.
5. OG and EA time is interdependent. Ideally OG time is shortened (5-15 Secs) and EA (6-8 mints) is increased by which eosin displaces OG.
6. Post-OG and post-EA alcohol is replaced by 0.5% of acetic acid. Acid pH keeps acid dyes in cells as 95% alcohol. In addition, dilute acetic acid solution is too weak to remove hematoxylin as HCl used to differentiate in regressive procedure. Plain water if used alone extracts counterstain dyes rapidly and should not be used. Acetic acid rinses do not require special disposal and is 10 times less concentrated than vinegar and vinegar has pH 3.4-2.4 which is generally considered corrosive. 0.5% acetic acid is not considered to be characteristic hazardous waste and hence not covered by the resource conservation and recovery act.
7. Water scavenging aluminosilicate beads i.e. molecular sieves are added to xylene by which xylene is filtered and reused indefinitely. The beads absorb water. However, the absolute alcohol immediately preceding xylene must be maintained colour free to avoid contamination of xylene with dyes (Bales, 2006; Catherine M Keebler, 2008,; Gill, 2006).

The advantages of enviro-Pap stain are; decrease lab annual staining reagent purchase, stains and reagents are readily available, eliminate eight 95% ethanol baths replacing 0.5% acetic acid and thus decrease the cost, chemical bluing agent excluded, decrease xylene disposable cost, can be easily implemented in any cytology lab, can be extended to histopathology lab, high quality yield and reproducible staining results. The disadvantages are over staining of OG which can be prevented by monitoring staining time and quick shift of slides from OG bath to 0.5% acetic acid bath (within 5-15 secs) (Catherine M Keebler, 2008,; Gill, 2006).

REAP stain

It is rapid, economical, acetic acid, Pap stain (REAP) where the staining time is 3-4 mints. It was introduced by S.B. Dighe in 2005. Even though UFP and rapid pap takes less time for staining, it requires large quantity of alcohol and hence it is expensive. To overcome this, REAP stain was prepared where 1% acetic acid replaces alcohol except for first step of fixation and last step of dehydration where absolute alcohol is used as in routine Pap stain. Hence, acetic acid act as mild dehydrating agent, nuclear fixative, increase staining intensity of nucleus, preserve colour of stain, rapid staining, cheap and easily available (Asthana A, 2014; Izhar et al., 2014; RoyBiswas et al., 2008). The technique of staining is shown in Figure 11. Harris Hematoxylin is preheated to 60 deg Centigrade.[2] The slides are blotted after each step (Papanicolaou, 1942). The cytomorphology with REAP stain is shown in Figure 12.

The modification in REAP stain are 95% ethanol is replaced by 1% acetic acid and descending grade of ethanol or 2 steps of dehydrating ethanol is replaced by one step acetic acid. Harris hematoxylin is heated in water bath to 60 deg C for rapid penetration of stain. No acid differentiation done. Blueing is done in ordinary tap water and not in Scott's tap water with less time. Time for cytoplasmic stain is reduced and concentration is 4 times more. Methanol is used for final dehydration (Asthana A, 2014).

OG-6 and EA36 are alcohol (ethanol) based stain. In routine Pap stain, after cytoplasmic staining, alcohol diffuses into dehydrating medium (ethanol) resulting in decreased staining intensity. In REAP stain 1% acetic acid reacts with ethanol of cytoplasmic stain and results in ethylacetate and water. Water is removed out of cell. The ester (ethyl acetate) complexes with cytoplasmic stain and is deposited in the cells preserving staining intensity. In routine pap stain following dehydration with ethanol, some ethanol enters and remains in the cell after clearing. This ethanol following mounting with DPX and sometime later dissolves cytoplasmic and nuclear stain which percolates into DPX. Hence color preservation does not last long. In REAP ethyl acetate preserves cytoplasmic staining and acetic acid being a nuclear fixative preserves nuclear stain (Asthana A, 2014).

The advantages of REAP stain are; better than routine Pap stain, excellent nuclear / cytoplasmic staining with better color intensity / transparency, cost

effective as acetic acid replaces costly ethanol (25% of total cost of standard Pap stain), long term color preservation (more than 1 year without fading), quick procedure, staining of non epithelial cells like RBC/WBC/bacteria are well preserved, nuclear /chromatin/ nucleolus details are clear/crisp, cynophilia / eosinophilia of the cells are comparable to Pap stain and lyses RBC with no change in epithelial cell morphology. Because of its cost effectiveness it can be used as a suitable alternative to standard pap stain in mass cervical cancer screening in developing countries (Asthana A, 2014; RoyBiswas et al., 2008).

The disadvantages of REAP stain are; it gives suboptimal staining in thick smears especially in cell clusters because of poor penetration of stain. Some authors are of opinion that REAP stain gives inferior staining quality with poor preservation when slides are kept for more than 6 months. Also stains can be better used in resource limited settings where cost factor is an issue and not for research purpose or tertiary setup (Izhar et al., 2014).

Cytocolor Merck

(www.merkmilipore.com/IN/en/producy/CYTOCOLOR)

Pap stain has been modified by Merck. The staining technique is show in Figure 13 and cytomorphology in Figure 14. Smears are fixed with spray fixative or in ethanol for 30 minutes. Total time taken is short and it is 3 minutes. It is ideally used for gynecologic cytology and it gives information regarding integrity, hormonal status and vaginal flora. The stain does not have OG unlike conventional Pap stain and hence cytoplasm of mature and keratinized cells appears pink. In cervical smears, nuclei appear blue / dark violet / black, cyanophilic cytoplasm appear blue-green, eosinophilic cytoplasm pink, keratinized cytoplasm bright pink, micro-organisms blue-violet, Trichomonads grey-blue / grey-green and erythrocytes red. It can also be used in fine needle aspiration cytology, fluid effusions and excised specimens.

Further modifications of cytocolor is made, Neo-cytocolor where the staining procedure is same except last two steps of xylene is replaced by two steps of Neo-clear which has same reproducibility and speed, in addition more user / environmentally friendly. Neo-clear is an aliphatic hydrocarbon which is virtually odorless, gives same results as xylene, has low rate of evaporation and gives good staining results.

CONCLUSION

The pap stain and its staining procedure have undergone a lot of changes since 1947. There are different formulations of stain available by vendors and different procedure taught to cytotechnologists. Hence each lab should have their staining protocols and it should be standardized.

References

Asthana A, S.A. (2014). Comparison of the routine Papanicolaou staining technique with the rapid, economic, acetic acid, Papanicolaou (REAP) technique. *IJMDS* 3(2), 484-489.

Bales, C.E. (2006). Laboratory Techniques. In: Leopold G Koss, Myron R Melamed (Eds). Koss' Diagnostic Cytology and its Histopathologic Basis, Vol 2, 5 th edn (Philadelphia: Lippincott Williams & Wilkins).

Catherine M Keebler, M.F. (2008,). Cytopreparatory Techniques. In: Masluce Bibbo, David C Wilbur (Eds). Comprehensive Cytopathology., 3rd edn (Philadelphia: Saunders (Elsevier)).

Choudhary, P., Sudhamani, S., Pandit, A., and Kiri, V. (2012). Comparison of modified ultrafast Papanicolaou stain with the standard rapid Papanicolaou stain in cytology of various organs. *Journal of Cytology* 29, 241.

Gill, G.W. (2006). Enviro-Pap: an environmentally friendly, economical, and effective Pap stain. *Laboratory Medicine* 37, 105-108.

GN, P. (1960). Memorandum on Staining, in Atlas of Exfoliative Cytology. *Harvard University Press, Cambridge MA,*, 12.

Izhar, S., Kaur, R., and Masih, K. (2014). Efficacy of rapid, economical, acetic acid, Papanicolaou stain in cervical smears as an alternative to conventional Papanicolaou stain. *Journal of cytology/Indian Academy of Cytologists* 31, 154.

Kamal, M., Bodele, A., Munshi, M., Bobhate, S., and Kher, A. (2000). Efficacy of a modified Ultra Fast Papanicolaou (UFP) stain for breast aspirates. *Indian journal of pathology &microbiology* 43, 417-421.

Papanicolaou, G.N. (1942). A new procedure for staining vaginal smears. *Science* 95, 438-439.

Papanicolaou, G.N. (1954). Atlas of exfoliative cytology. *Atlas of exfoliative cytology*.

RoyBiswas, R., Paral, C.C., Dey, R., and Biswas, S.C. (2008). Rapid Economic, Acetic Acid, Papanicolaou Stain (REAP)-Is it suitable alternative to standard PAP stain?

http://www.merkmilipore.com/IN/en/producy/CYTOCOLOR.

Yang, G., and Alvarez, I.I. (1994). Ultrafast Papanicolaou stain. An alternative preparation for fine needle aspiration cytology. *Acta cytologica* 39, 55-60.

Yang, G.C., Papellas, J., Wu, H.C., and Waisman, J. (2001). Application of ultrafast Papanicolaou stain to body fluid cytology. *Acta cytologica* 45, 180-185.

Cite this article as:

Raju, K. (2016). Evolution of Pap Stain. *Biomedical Research And Therapy, 3*(2), 490-500