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Efficacy of Methylene Blue and Papanicolaou Stain in Sex Determination-A Comparative Study

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ABSTRACT

Introduction: Exfoliative cytology of epithelial cells is a non-invasive easy method for the collection of samples and the examination of the cells. The cells are collected by brushing, scraping, or by any abrasive method. Barr body or sex chromatins are a condensation of chromatin present at the nucleus of cells in female individuals. Diagnostic cytology is an important element of the criminal investigation of assault, robbery, sexual assaults, criminal cases, sports, etc.

Aim: The study aims to find the efficacy of methylene blue and Papanicolaou staining in sex determination with the help of Barr bodies.

Materials and methods: The smears were collected from 10 healthy males and 10 healthy females. First, the individual participant was asked to gargle the mouth with water, then the smear was collected with the help of the wooden spatula. The wooden spatula was scraped against the buccal mucosa from the epithelial layer and was smeared in two glass slides from each individual with a single Stoke. The smear was air-dried and fixed in 95% isopropyl alcohol in a Coplin jar for 40 minutes followed by Papanicolaou staining and methylene blue staining and the slides were mounted in DPX and observed under 40x and number of barr bodies was counted. SPSS software was used and a chi square test was used.

Results: 97 percent sensitivity was shown with Papanicolaou staining when compared with Methylene Blue. 98 percent specificity was shown with Papanicolaou staining when compared with Methylene Blue which showed 94 percent. The p value is 0.03 which is less than 0.05 which shows the results are significant.

Conclusion: From the study we can conclude that both Papanicolaou stains and methylene blue staining techniques are good in identification of barr bodies. 97 percent accuracy was shown with Papanicolaou staining when compared with Methylene Blue which showed 96%. We can conclude that Papanicolaou stain is more efficient in the identification of Barr bodies than that of methylene blue.

Key words: Barr bodies, Papanicolaou stain, Methylene blue, Efficacy, Innovative technique

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INTRODUCTION

Diagnostic cytology is the science of study of the cells that are from epithelial cell surfaces or different tissues. Diagnostic cytology has the advantages of being a non-invasive, straightforward procedure that aids in faster reporting, as well as being inexpensive and popular with people all over the world facilitating cancer screenings in the field [1]. This can be carried out by different methods including collection and examination of exfoliated cells such as sputum, body fluids urine, etc., [2]. The cells are collected by brushing, scraping, or by any abrasive method. In females, the sex chromatin, also known as the Barr body, is a chromatin condensation found in the nucleus of cells. There are males heterogametic (XY) and female homogametic (XX). Compensation of dosage is accomplished by spontaneous inactivation of one of the two X chromosomes. In species with XY sex-determination, a Barr body (named after discoverer Murray Barr) is an inert X chromosome in a cell with more than one X chromosome, made dormant in a phenomenon called lyonization (including humans). In humans with more than one X chromosome, the number of Barr bodies visible at interphase is always one fewer than the total number of X chromosomes [3,4]. In typical female somatic cells, it acts as a facultative heterochromatin dark-stained peripheral nuclear structure evident during interphase.

Diagnostic cytology is an important element of the criminal investigation of assault, robbery and sexual assaults, criminal cases [5,6]. Buccal smears help to classify the sex and identify the person on various occasions. Gender determination of a living person is required in doubtful cases like in sports, with altered physical and sexual features, and to decide certain civil rights reserved for one sex [7-9]. In cases of sexual offenses, the buccal mucosal cells along with saliva stains

are found in various parts of the body and also at the scene of a crime.

In previous studies, many authors have done similar studies to show the sex determination done with help of buccal mucosa scrapping with the help of various staining methods. Gender determination was done by various methods such as karyotyping, polymerase chain reaction (PCR), fluorescent body (Y chromatin) demonstration in male somatic cells, Davidson body in female neutrophils, and Barr body in female somatic cells [10]. Nagamori et al. [3] did a similar study in gender determination using Barr bodies using Giemsa and methylene blue stain. In that article, the author has compared the efficacy, sensitivity, and specificity of each stain and compared it. Many authors have done similar studies with various stains and compared different parameters in finding the Barr bodies. In most of the study, the main challenges faced by the author were to identify the Barr bodies in the specimen

The buccal smear method is widely popular as the smear can be taken easily without major inconvenience and it is a non-invasive method. Different stains like Papanicolaou, orcein, Guard, cresyl violet, and many other stains, as well as fluorescent staining methods, have been used to demonstrate the presence of Barr bodies.

A Barr body is an intense staining body, approximately 1 μm in diameter, which is flat-convex or triangular [11,12]. It is most commonly located on the side of the nuclear membrane and can be seen on the periphery of the nucleus. In buccal epithelial cells, pulp fibroblasts, cervical cells, skin and hair, sex chromatin has been studied [13]. The oral process is commonly used as specimens can be collected with limited inconvenience [14]. Various stains, such as Papanicolaou (PAP), orcein, Feulgen, Patrol, Cresyl Violet as well as fluorescent staining techniques, have been used to show the presence of Barr bodies. Giemsa is one such strain that is inexpensive and results easily when compared to other strains.

This research was mainly done to find the efficacy of two different stains in identifying the Barr bodies from the buccal mucosal scraping and compare the efficacy, sensitivity, and specificity in identifying the Barr bodies from the specimen [15], from this study we can find the efficacy, sensitivity, and specificity of both methylene blue and Papanicolaou stain with the help of the research. The study aims to find the efficacy of methylene blue and Papanicolaou staining in sex determination with the help of Barr bodies.

Methylene blue which is similar to toluidine blue is being widely used in the medicinal field. This binds to the DNA and RNA material by its acid-binding capacity that has a high affinity towards nucleic acid so binds with DNA and RNA content of the cell/tissue [16]. Papanicolaou stain(PAP) was given by George Papanicolaou. It is a multi-coloured stain that is polychromatic and can stain discrete cellular components and was first used in cervical smear & fine-needle aspiration. Our team has extensive knowledge and research experience that has translate into high quality publications [17-36].

The study aims to find the efficacy of methylene blue and Papanicolaou staining in sex determination with the help of Barr bodies.

MATERIAL AND METHOD

The oral smear was obtained from the mucosa of healthy individuals. The oral smears were collected from the participant with their consent and ethical approval was obtained from Saveetha dental college and the ethical number is IHEC/SDC/BDS/1982/01. The advantage of collecting samples from buccal mucosa is that it is a non-invasive, cost-efficient method. Improper staining technique and overlapping of cells can lead to difficulty in Barr body identification. The smears were collected from 10 healthy females.

First, the individual participant was asked to gargle the mouth with water, and then the smear was collected with the help of a wooden spatula. The wooden spatula was scraped against the buccal mucosa from the epithelial layer and was smeared in two glass slides from each individual with a single stroke. The smear was air-dried and fixed in 95% isopropyl alcohol in a Coplin jar for 40 minutes followed by Papanicolaou staining and methylene blue staining.

In Papanicolaou Staining, first, the smear was hydrated for 3-5mins in tap water and excess water was blotted out and then a few drops of nuclear stain rapid Papanicolaou are added to cover the smear and kept for 60 seconds undisturbed and was washed in running water, 3-5 drops of wash buffer was added for 20 seconds and washed and excess water was blotted out, then dehydrate is added and kept undisturbed for 60 seconds and should not be washed and then few drops of cytoplasm stain are added such that the stain covers the slide and the smear and left undisturbed for 60 seconds and then wash the slide in water and blot out the excess water and left to dry and mounted with DPX.

In Methylene blue staining, first, the smear is dried and then the slide is flooded with methylene blue and left undisturbed for five to ten minutes and the slides are washed under tap water and the excess water is blotted out and then to the smear about 1-2 drops of glycerine is added and left undisturbed for 1 minute and then it is left to dry and mounted with DPX.

After the staining process is completed the smear in the slides were observed under a microscope under 40x respectively. The following results were obtained from the observations on the oral smear following the staining of Papanicolaou and Methylene Blue. This study involved a total of 20 samples. There were 10 participants in both male and female groups for each stain.

The data was collected after being examined through the microscope, the collected data was put in SPSS statistical software and the statistical test done was chi square test. The dependent variable for the study is sex and the independent variable is age. The inclusion criteria for the

study are healthy individuals and the exclusion criteria are individuals with lesion, cancer in buccal mucosa.

RESULTS

Results are explained in the form of Figures and Tables (Figures 1 to Figure 6) (Table 1).

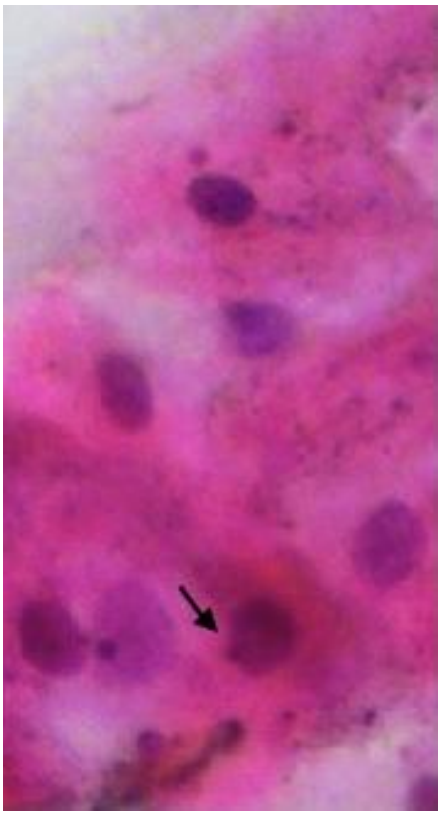

Figure 1: This image shows the presence of barr bodies in papanicolaou staining (black arrow). The Barr bodies are focused on 100x oil immersion.

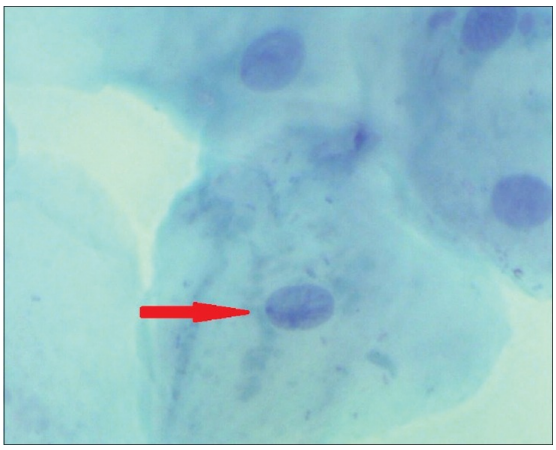

Figure 2: This image shows the presence of Barr bodies in methylene blue staining (red arrow). The Barr bodies are focused on 100x oil immersion.

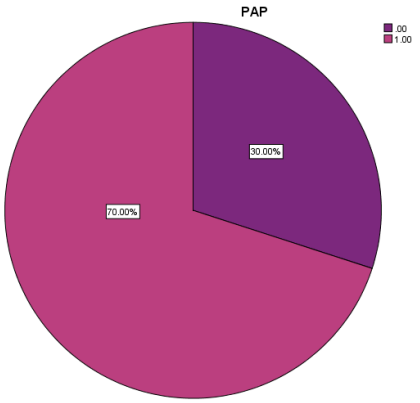

Figure 3: This pie chart depicts the papanicolaou staining frequency of identification of barr bodies. Red color represents barr bodies present in the smear, 70.00% of the sample shows Barr bodies. Purple color represents Barr bodies present in the smear, 30.00% of the sample do not show Barr bodies. The p value is 0.03 which is less than 0.05 with which we can conclude the data is significant.

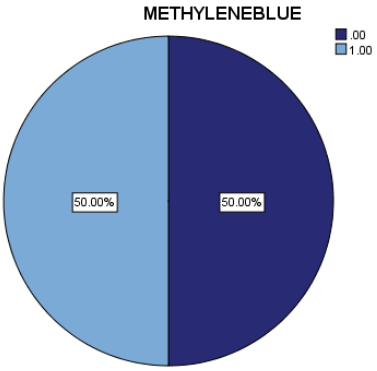

Figure 4: this pie chart depicts the methylene blue staining frequency of identification of barr bodies. 1.00- represents barr bodies present in the smear, 50.00% of the sample shows barr bodies (blue). 0.00- represents barr bodies present in the smear, 50.00% of the sample do not show barr bodies (deep blue).

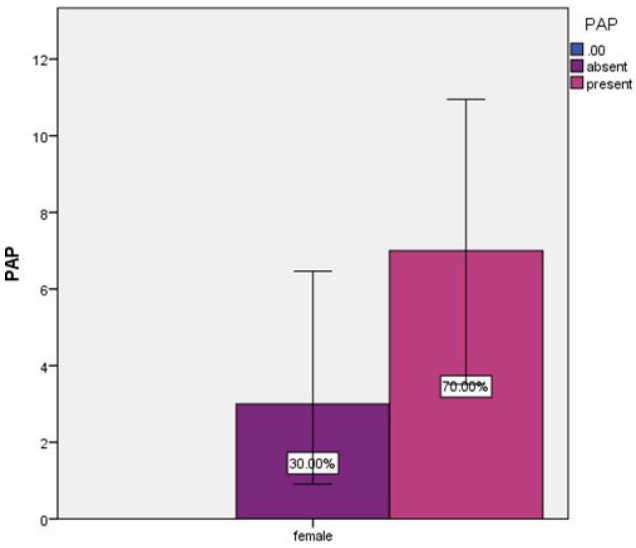

Figure 5: The above graph depicts the amount of error between the female population and the number of Barr bodies in PAP smears with X Axis indicates the number of female participants and Y Axis represents the PAP smear in relation to the number of presence and absence of Barr body. 30% of the population showed absence of a barr body and 70% of the population showed a presence of a barr body.

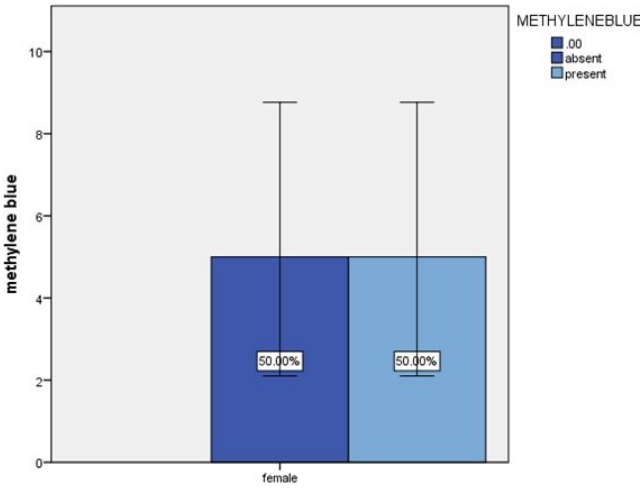

Figure 6: The above graph depict the amount of error between the female population and the number of Barr body in methylene blue stained smears with X axis indicates the number of female population and Y Axis represents the methylene blue smear in relation to the number of presence and absence of barr body. 50% of the population showed absence of a barr body and 50% of the population showed the presence of a barr body.

Table 1: This table depicts the comparison of sensitivity, specificity, and accuracy of both the stains.

Data analysis	Papanicolaou staining	Methylene blue
Sensitivity	97%	96%
Specificity	98%	94%
Accuracy	97%	95%

DISCUSSION

From the observation of the buccal smear after staining with Papanicolaou and methylene blue staining the following results were observed. The observed mean age of the participants is ± 23.7 years in age. Figure 3 shows the frequency of the Barr bodies in Papanicolaou staining. 70.00% of the smear sample showed the presence of Barr bodies and the rest of the smear samples, 30.00%, did not show the presence of Barr bodies in the sample. A similar study was done by Baby et al. [37,38] and the studies showed similar results in which Papanicolaou staining showed a higher presence of Barr body when compared with Acriflavine Schiff stain in his study. Figure 4 shows the presence of Barr bodies in Methylene Blue staining. The stained smears were examined and 50.00% of the smear samples showed the presence of Barr bodies and the rest of the smear sample that is 50.00% of the smear sample, did not show the presence of the Barr bodies. A similar study was done by Rebhun et al. [39] in which methylene blue showed a similar outcome in frequency of identification of Barr bodies. Table 1 shows the comparison of sensitivity, specificity, and accuracy of both methylene blue and Papanicolaou stain. The sensitivity of the Papanicolaou stain is 97% and the methylene blue stain is 96%, this shows that the Papanicolaou stain has more sensitivity than that of the methylene blue stain. The specificity of the Papanicolaou stain is 98% and the specificity of methylene blue is 94%, this shows that Papanicolaou stains show more specificity than that of methylene blue staining. From table 1, the accuracy of the Papanicolaou staining is 97% and methylene blue staining is 95%, this shows that Papanicolaou staining [40] is more accurate than that of methylene blue staining.

From that Table 1, we can say that Papanicolaou staining has more sensitivity, specificity, and accuracy than that of methylene blue stain. Similar studies have been done on the identification of Barr bodies using various stains. A similar study done by Datar et al. [11] on identification and the studies shows similar results for Papanicolaou stain as this study, Papanicolaou staining showed high accuracy and specificity when compared to other stains techniques. Another study by Britto et al. and Aziz et al. [5,41] showed that Papanicolaou stain shows less specificity and accuracy when compared to acridine orange stain, this study opposed the finding of this study. From the previous literature, many authors have done similar research on different dyes and all the dyes helps

in the identification of Barr bodies, based on the referred and previous articles overall Papanicolaou stain has more specificity, sensitivity, and accuracy when compared to methylene blue but other stains like acridine orange and other stains have more specificity, sensitivity and accuracy than the Papanicolaou stain.

The Figure 5 graph depicts the amount of error between the female population and the number of Barr bodies in PAP smears with X Axis indicates the number of female participants and Y Axis represents the PAP smear in relation to the number of presence and absence of Barr body. 30% of the population showed absence of a barr body and 70% of the population showed a presence of a barr body and in figure 6 graph depict the amount of error between the female population and the number of Barr body in methylene blue stained smears with X axis indicates the number of female population and Y Axis represents the methylene blue smear in relation to the number of presence and absence of barr body. 50% of the population showed absence of a barr body and 50% of the population showed the presence of a barr body.

The main limitation of this study is that the sample size of the study is small and the smear samples were taken only from that of the female and staining was compared only between Papanicolaou stain and methylene blue, Improper staining technique and overlapping of cells can lead to difficulty in Barr body identification under the microscope.

Taking the shortcomings, the advantages of Papanicolaou and methylene blue staining are their relative simplicity, cost-efficient, less time consumption. However, there is a great advantage, rapid diagnosis easily accessible when prompt diagnosis is essential. For the future scope of this study, using various staining techniques we can identify the specificity, sensitivity and accuracy, and other parameters of each stain and correlate the data of different parameters for each stain and come up with a standard staining technique that has the highest accuracy, specificity and sensitivity and average highest in other parameters.

CONCLUSION

From our study done we can conclude the sex of the individual can easily be identified by determining the percentage of Barr-body-positive cells. In this study, sex determination with Barr bodies in buccal smears was found to be a direct method with up to 97 percent accuracy when with Papanicolaou staining when compared with Methylene Blue, this method is less time consuming, inexpensive, reliable, and repeatable. We can conclude that Papanicolaou stain is more efficient in the identification of Barr bodies than that of methylene blue.

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CONFLICT OF INTEREST

The author declares that there was no conflict of interest in the present study.

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