ASSESSMENT OF GENDER BUCCAL BARR BODIES USING PAPANICOLAU, ACETO ORCEIN AND FEULGEN STAIN AMONG SOUTH INDIAN POPULATION- A CYTOLOGICAL STUDY

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Abstract :

Background: Determination of sex is very important in forensic medicine. Barr bodies are unique chromatin structures formed in nuclei of the mammalian females as a means of sex chromosome dosage compensation. Special stains on buccal scrapes for detection of Barr bodies for sex determination Hence this, preliminary study was aimed to identify gender using buccal Barr bodies to compare the staining efficacy of papanicolau, aceto orcein stain and feulgen stain.**Methodology :** Two samples of buccal smears, collected from fifty males and fifty females in the age group of 16–60 years were used to demonstrate Barr bodies using AO, feugel stain and PAP stains, respectively. After staining, Barr bodies were detected in male and female and compared using chi square test. **Results :** The reults compared Barr body positive cells in males and females using Papanicolaou stain (PAP), AO and feugel stain respectively are statistically significant.(<0.001). The intensity of Barr body positive cells in males and females using Papanicolaou stain , aceto-orcein stain and feulgen stain showed statistically significant difference.(<0.001).**Conclusion:** Sex determination using Barr bodies in buccal scrapes is a simple method providing up to 95–98% accuracy. The PAP squash method is a rapid, economical, accurate, and reproducible method, comparable to AO, for the detection of Barr bodies. This study provides relevant information regarding the diagnostic accuracy of this method in a representative sample of Indians.

INTRODUCTION

Sex determination results in the development of individuals with characteristics that allow them to be identified as males, females, or in some cases, hermaphrodites.[1]Determination of sex is very

important in forensic medicine. But very often, the available skeleton resource in forensic identification of sex of the individual becomes a challenging task owing to the deficiency of the substance for specimen analysis.

Sex determination can be done either by morphometric analysis (of the tooth, skull and other soft tissues of oral and paraoral region) or by using molecular analysis[2,3] which involves conventional technique like Polymerase Chain Reaction (PCR) and karyotyping which are expensive and time consuming.In such cases, the tooth which remains intact can be of significance in forensic identification of sex of the individual. Barr and Bertram determined that there exists difference between male and female cells by presence of Barr body in females[4].

Sex of the individual can be determined based on the morphology of canines. Apart from this method, it can also be determined by using X and Y chromosomes in the cells which are inactive. X chromatin in its inactivated form is present as a mass against the nuclear membrane in females is known as Barr body as it was first named by Barr and Bertem (1949)[5]. These Barr bodies are present in 40% of females who are considered as chromatin positive and absent in males who are considered as chromatin negative. Similar to X chromosome, Y chromosome (F bodies) can also be studied for sex determination of males. Both X and Y chromosomes are found to be present during inter phase of the cell cycle[6.]

Barr bodies are unique chromatin structures formed in nuclei of the mammalian females as a means of sex chromosome dosage compensation. Special stains on buccal scrapes for detection of Barr bodies for sex determination has been used as an important tool in forensic analysis as it provides 95-98% accuracy, making it a significant accessory for sex determination.[7]

Though many stains are used in identifying Barr bodies, only one study is reported in the literature using acriflavine (AF) Schiff on buccal smears.[8] The Papanicolau stain is one of the most widely used stains in cytology,[9] where it is used to aid pathologists in making a diagnosis. Pap staining is usually associated with cytopathology in which loose cells are examined, but the stain has also been modified and used on tissue slices. Feulgen stain is considered to be specific for Barr body and a better stain for sex chromatin as it is DNA specific and also is negative for nucleus and Nissl bodies which can be confused with Barr body/sex chromatin.

Aceto-orcein (AO) is economical and yields prompt results, especially when the squash technique is used. Few studies have been done employing AO [10,11] , and none have compared the efficacies of AO, PAP and feulgen for the identification of Barr bodies. Hence this, preliminary study was aimed to identify gender using buccal Barr bodies to compare the staining efficacy of papanicolau, aceto orcein stain and feulgen stain.

MATERIALS AND METHODS:

The present study was conducted at Department of Oral Pathology and Microbiology, Vivekanandha dental college for women.

SELECTION OF STUDY PARTICIPANTS:

A total of fifty men and fifty women in the age group of 16–60 years were selected randomly for the study. A written informed consent was obtained from all the participants. The participants were first asked to rinse the mouth with water. Two buccal mucosal smears were collected from every participant by scraping with a clean flat wooden spatula after labelling the slide. The slides were air dried and fixed in 95% isopropyl alcohol for 30 min before staining.

PREPARATION OF SMEARS- CYTOLOGICAL PREPARATIONS:

Exfoliated buccal epithelial cells were obtained from the middle part of the inner cheek using a moistened wooden spatula. Prior to buccal epithelial cell collection, the subjects rinsed their mouth thoroughly with water to remove food particles and unwanted debris.

i. Smears of the cells were prepared on pre-cleaned grease free microscopic glass slides. Two slides were prepared for each individual. Code number was assigned to each slide (figure 2).

ii. These slides were transported to the laboratory in ice where they were air dried and hydrolyzed in 1N HCl at 60oC for 8 minutes and rinsed in water.

PAPANICOLAOU STAINING METHOD:

The samples were stained in Harris's hematoxylin after rehydration, followed by dehydration in isopropyl alcohol and stained in OG 6 for 2 min. After rinsing in 95% isopropyl alcohol twice for 2 min each, they are stained in EA 50 for 3 min followed by rinse in 95% isopropyl alcohol for 1 minute.[2]

ACETO-ORCEIN STAIN:

Aceto-orcein stain turns chromosomes a purple-red colour. This stain can be prepared from powder or purchased as solution; staining is best with freshly prepared stain as, over time, the stain precipitates and changes colour from a deep red or burgundy colour to a brownish colour, which gives results that are equally good in fresh and permanent preparations. A 45% acetic and 1% orcein content is recommended as a standard solution.

PROCEDURE

(1) Aceto-orcein stain: Aceto-orcein stain Acetic acid : 60 ml dH2O : 40 ml Orcein : 1 gm A 60% acetic acid solution was prepared by dissolving 60 ml of acetic acid in 40 ml distilled water to prepare the working aceto orcein stain ,dissolved 1 gm of orcein in 50 ml of 60% acetic acid solution by slow heating. This stain was cooled, filtered and stored for the further use.
(2) Then slides were stained with Aceto-orcein (2% in 60% acetic acid) for 20 minutes at 40oC and washed in ethanol and distilled water for two to three times. iv. The slides were counter-stained with 0.1% Fast Green solution for 15 minutes and rinsed in ethanol and distilled water. v. After that the slides were air-dried.

FEULGEN STAIN

PROCEDURE

1.Fixed material is treated for 8-10 min with 1N HCl in a water bath or oven at 60°C.

2.Afterwards, the material is immediately transferred into Schiff's reagent at room temperature (for at least 30 min or until the tissue stains deep purple). The material is then squashed in acetocarmine or aceto-orcein.

3.It is recommended that the material be analyzed the same day, however, it can be kept at 4°C for a several days if necessary.

4.Acid hydrolysis removes purin bases from the DNA, thereby unmasking free aldehyde groups. The aldehyde groups then react with Schiff's reagent, which results in the purple staining.

Barr body count

Cells with the intact nuclear membrane, without any crenation or depression, and the chromatins without clumps were selected and counted. The frequency of Barr body was examined by observing 100 nuclei per specimen under oil immersion. The Barr bodies that were found directly inside and attaching to the nuclear membrane were regarded as positive. Data obtained were compiled systematically in Microsoft Excel spreadsheet (ANNEXURE – IV).Statistical analysis was performed using statistical package for social sciences software (IBM Corp.Released 2011. IBM statistics for Windows, Version 20.0. Armonk, NY: IBM Corp). Descriptive statistics were performed.

RESULTS

The number of patients and the detection of Barr bodies which appeared as dumbbell shaped extension from the lobed nucleus in neutrophils of peripheral smear as seen in figure and hyperchromatic dot in the peripherary of the nucleus or in the nuclear memebrane, in the buccal smear using Papanicolaou stain (PAP), aceto-orcein stain, feulgen stain is seen in figure 1,2,3 respectively.

FIGURE 1: Barr body count using Papanicolau stain

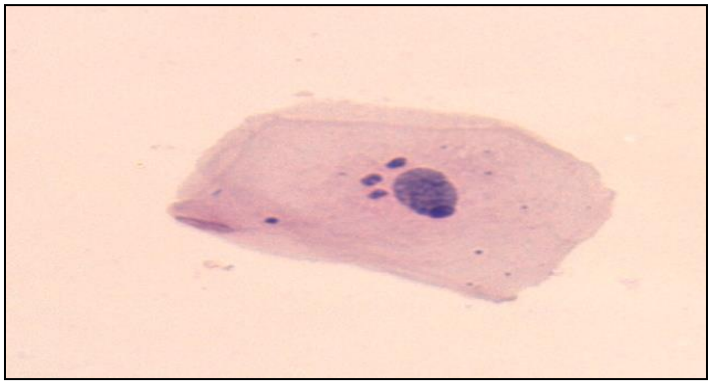


FIGURE 2: Barr body count using Aceto Orcein stain

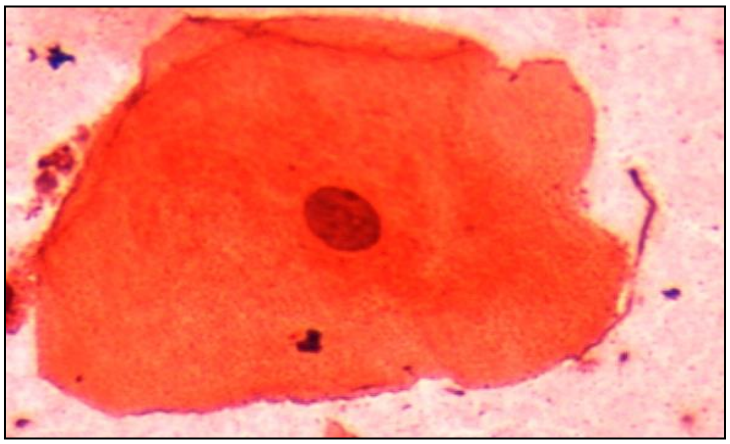
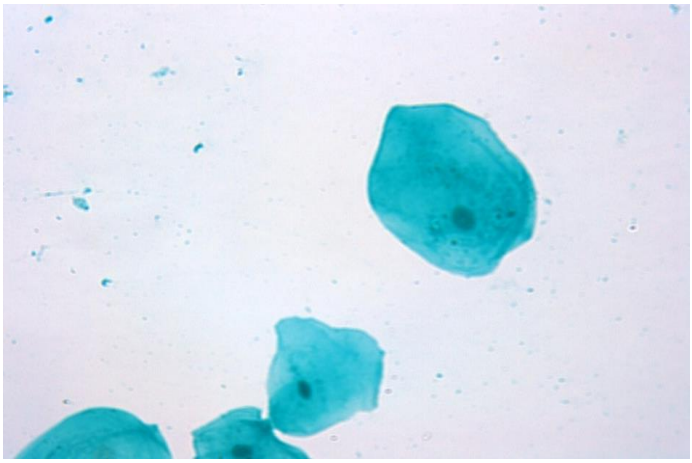


FIGURE 3 : Barr body count using Feulgen stain



The detection of Barr bodies in buccal scrapes using **Papanicolaou stain** (PAP) is graded as presented in table 1 and using **Aceto-orcein** is presented in table 2 and usion **Feulgen stain** is presented in table 3. There was a statistically significant diffference was found among males and females using **Papanicolaou stain (PAP) (p value <0.001*), aceto-orcein stain (p value <0.001*), feulgen stain (p value <0.001*)** .

TABLE 1: Comparison of Barr body positive cells in males and females using Papanicolaou stain (PAP).

Sex	**Total no. Of cases**	**Total no of smears**	**Present (%)**	**Absent (%)**	**χ2 value**	**p-value**
Male	50	50	0 (0%)	50 (100%)	33.333	<0.001*
Female	50	50	40 (80%)	10 (20%)		
Total	100	100	40 (40%)	60 (60%)		

Chi square test satistically significant p<0.05*

TABLE 2: Comparison of Barr body positive cells in males and females using aceto-orcein stain.

Sex	**Total no. Of cases**	**Total no. of smears**	**Present (%)**	**Absent (%)**	**χ2 value**	**p-value**
Male	50	50	0 (0%)	50 (100%)	42.593	<0.001*
Female	50	50	46 (92%)	4 (8%)		
Total	100	100	46 (46%)	54 (54%)		

Chi square test satistically significant p<0.05*

TABLE 3: Comparison of Barr body positive cells in males and females using feulgen stain.

Sex	**Total no. Of cases**	**Total no. of smears**	**Present (%)**	**Absent (%)**	**χ2 value**	**p-value**
Male	50	50	0 (0%)	50 (100%)	46.573	**<0.001***
Female	50	50	41 (92%)	9 (18%)		
Total	100	100	44 (44%)	56 (56%)		

Chi square test satistically significant p<0.05*

Intensity of detection of Barr bodies in buccal scrapes using **Papanicolaou stain (PAP), aceto-orcein stain (AO) and feulgen stain** is graded as presented in table 4.invetigation reveals that there was a dynamic cganges was noted in intensity when increase the concentration of three different types of stain. Strong PAP stain shows higher intensity (76%) when compare with others.

Table 4: Comparison of intensity of Barr body positive cells in males and females using Papanicolaou stain , aceto-orcein stain and feulgen stain.

Stain	**Weak** **+** **n (%)**	**Moderate** **++** **n (%)**	**Strong** **+++** **n (%)**	**X^2 value**	**p-value**

PAP	2 (2%)	22 (22%)	76 (76%)	44.939	**<0.001***
Aceto-orcein	26 (26%)	44 (44%)	30 (30%)		
feulgen stain	22(22%)	42(42%)	36(36%)		

Chi square test satistically significant p<0.05*

The sensitivity values for the detection of Barr bodies using PAP stain AO stain and feulgen stain were calculated as 92% and 80%,and 85% respectively. The specificity values for the detection of Barr bodies using PAP stain AO stain and feulgen stain were calculated as 100% and 100%, and 80% respectively. Accuracy for the detection of Barr bodies using PAP stain AO stain and feulgen stain were calculated as 92% and 80%,and 86% respectively were tabulated as present in table 5.

Table 5: Sensitivity, specificity and accuracy of Papanicolaou stain , aceto-orcein stain and feulgen stain

Test	**Papanicolaou stain (**PAP)	**Aceto-orcein**(AO)	**Feulgen stain**
Sensitivity	92 % (73.97 to 99.02)	80% (59.30 to 93.17)	85% (60.20 to 91.57)
Specificity	100% (86.28 to 100)	100% (86.28 to 100)	80% (76.18 to 100)
Accuracy	96% (86.29 to 99.51)	90% (78.19 to 96.67)	86% (86.58 to 93.36)

DISCUSSION

In the presence study, the mean percentages of Barr bodies were consistently higher in females than in males which is similar with other studies. Identification of Barr body in males was significantly narrower in range than in females.

The sex of an individual can be determined by a number of ways. Demonstration of nuclear sex plays a vital role as far as sexing of the individual is concerned. Nuclear sex can be demonstrated by the study of: Karyotyping: direct study of type of sex chromosome in the cell by culture of the cell. This is expensive and is not feasible in my institution Vivekanandha dental college for women. Fluorescent body (Y chromatin): a demonstration of nuclear fluorescent bodies indicates male. This requires special stain and fluorescence microscope.

Barr bodies are feulgen positive, heteropyknotic, basophilic, intranuclear structures, seen in mammalian cells during interphase. Since they are nuclear structures and all nuclear structures are known to fluoresce, Barr bodies also fluoresce. Most often, they are noticed as densely stained condensed chromatin masses adjacent to the nuclear membrane. In some cells (especially

neurons), they can be observed adjacent to the nucleolus or even free in the nucleoplasm. They can be plano-convex, biconvex, triangular, spherical, or rectangular in shape when observed under ordinary microscope in oil immersion or high power. Sometimes, they resemble the letter V, W, S, or X under electron microscope. They measure about 0.8 to 1.1 μm in diameter.[2,3,4]

The buccal smear technique to identify sex was first introduced by Moore and Barr in 1955. The process of inactivation of X chromatin is known as lyonization, the process named after the scientist Lyon. In 1961, Lyon outlined the X-inactivation, also known as the Lyon hypothesis. It states that only one of the X chromosomes is genetically active in females while the other X of either maternal or paternal origin undergoes random heteropyknosis and is inactive. This occurs at among all the cells of the blastocyst in females on or about the 16th day of embryonic life. Inactivation of the same X chromosome persists in all the cells derived from each precursor cell. Thus, normal women are in reality mosaics and have two populations of cells, one with an inactivated maternal X and the other with an inactivated paternal X.

Several stains have been used to demonstrate the presence of Barr bodies, including PAP, Aceto – orcein (AO), Feulgen , and Guard. The Feulgen and Guard stains are ideal but difficult to standardize, making them. Fluorescent stains can validate both X and Y chromosomes, but the stains are not routinely used and the fluorescent microscopy equipment needed for visualization may not be available in each and every situation.

PAP is a time-tested method and is widely used. Additionally, there was good intra- and inter observer correlation with both AO and PAP, emphasizing their reproducibility. However, diagnosis is rapid and allows easy scrutiny of the stained nucleus using the AO squash method. It is of great advantage when prompt diagnosis is essential. The slides do not deteriorate for several weeks. The fine nuclear details were effortlessly observed using this method and the Barr bodies were easily appreciated. These advantages of the AO squash method could make it a preferable method and we advocate its wider use.

Reddy et al. in their study using a small sample size of 20 males and females each observed a Barr body percentage of 18%–72%, and all the females expressed Barr bodies. Herein, the smears were stained with AO stain as in our study. In our study will showed lesser Barr body percentage of 4%–31% cells possibly due to the variation in the microscopic observation technique. In our study used fluorescent microscope as against leica microscope in the study done by Reddy et al., which yielded better results.[3]

Baby et al. carried out a study for the expression of Barr bodies using PAP and acriflavine Schiff (AF) staining methods. AF Schiff-stained positive cells ranged from 16% to 53% and PAP-stained positive cells ranged from 9% to 38% in females. In males, 0%–9% AF-positive Barr bodies and 0%–5% PAP-stained Barr bodies were recognized. However, in our study, in females, AO-stained positive cells ranged from 4% to 31% and PAP showed 3%–21%. In males, 0%–9% AO-positive Barr bodies and 0%–6% PAP-stained Barr bodies were recognized. The present study showed a better correlation with the above study using PAP stain.[2]

The present study correlated with the study done by Datar et al. in 2013, wherein the percentage of Barr bodies in AO-stained slides around 50 in females and 50 in males, whereas with PAP the ranges recorded were 50 in females and 50 in males ($P < 0.001$). In our study, in AO-stained

slides, the percentage of Barr bodies around 50 in females and from 50 in males, whereas with PAP the ranges recorded were 50 in females and 50 in males ($P < 0.001$). The present study showed sensitivity and specificity of around 92% and 100%,80% and 100%, 85% and 80% respectively, for AO and PAP and fuelgen stains used for detecting sex accurately, whereas the study by Datar et al. showed sensitivity and specificity of 98.3 and 95% for AO and PAP, respectively.[1]

The present study depicted a higher mean percentage of Barr bodies in females as compared to males. PAP confirmed a superior diagnostic precision for sex determination in comparison with AO and fuelgen. PAP has a greater benefit when accurate diagnosis is essential.

CONCLUSION

Sex determination using Barr bodies in buccal scrapes is a simple method providing up to 95–98% accuracy; this makes it a significant accessory to other methods of sex determination. The sensitivity values for the detection of Barr bodies using PAP stain AO stain and Feulgen stain were calculated as 92% and 80%, and 85% respectively. The specificity values for the detection of Barr bodies using PAP stain AO stain and Feulgen stain were calculated as 100% and 100%, and 80% respectively.The PAP squash method is a rapid, economical, accurate, and reproducible method, comparable to AO, for the detection of Barr bodies. This study provides relevant information regarding the diagnostic accuracy of this method in a representative sample of Indians.

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