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ORIGINAL ARTICLE

Rivalling efficacy of special stains in the identification of Barr bodies

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ABSTRACT:

Background: Gender determination becomes the first priority in process of identification of a person by a forensic investigator in case of mass disaster, crime investigations, and ethnic studies. Demonstration of nuclear sex plays a vital role in determining the gender of an individual. Barr bodies (BB) are known to arise from the inactivation of the X chromosome in a female somatic cell. They are basophilic structures with varying morphology measuring approximately 0.8 × 1.1 microns in the nucleus during interphase. Various nuclear stains such as Thionine, Papanicolaou, Feulgen, Cresyl-violet, Giemsa, Aceto-orcein, and acridine orange & Routine stains can validate Barr bodies.

Aim: To evaluate the efficacy of Feulgen, Giemsa, and H&E stains in the demonstration of Barr bodies.

Materials and Methods: Buccal smears were prepared from 25 males & 25 females. These smears were stained by Feulgen, Giemsa & Routine staining methods. The Barr body identification was determined by the presence of a darkly stained condensed area on nucleoplasm. The frequency of Barr body was examined by observing 100 nuclei per smear under a binocular light microscope at 100 magnification. For all BB-positive cases, Barr Body Index(BBI) was calculated and compared for the different stains used.

Results: Buccal cytological smears stained with Feulgen, Giemsa, and Routine stains. Samples with a presence of Barr bodies ≤2 were recorded as male and those with >2 were recorded as female under microscopic findings. The percentage of Barr bodies in Feulgen-stained slides ranged from 30.2 - 37.9 among females and 0 - 0.1 in males, Giemsa slides ranged 14.2 - 22.2 from among females and 0 - 0.3 in males, while with Routine stained slides ranges recorded were10.2 - 14.9 in females and 0 - 0.5 in males. The sensitivity of Feulgen, Giemsa, and Routine stain for detecting sex accurately was 100% and specificity was 100%, 98%, and 94% respectively.

Conclusion: Feulgen stain proved to be better than Giemsa and routine stain for visualizing Barr Bodies.

Keywords- Barr Body, Sex, Gender, Feulgen, Giemsa, Forensic Odontology

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INTRODUCTION:

The British Association for Forensic Odontology defined it as Branch of forensic medicine which in the interests of justice, deals with the proper examination, handling and presentation of dental evidence in a court of law[1].Gender determination helps in criminal investigations for identification of the person which can help in solving many cases of assault, theft, sexual offenses, etc. In natural disasters also it may help to identify the bodies[2].

The process and management of gender verification are enormously complex and pose challenges to the biological description of males and females. The gender of an individual can be determined in a number of ways. Demonstration of nuclear sex plays a vital role as far as sexing of the individual is concerned. It can be demonstrated by the study of Karyotyping, Fluorescent body (Y chromatin), and polymerase chain reaction. Among them, the Barr bodies (X –chromatin) detection method is advantageous in that it can be studied under an ordinary compound microscope with simple staining techniques[3].

Barr Body (BB) observation is possible in different cell types and is used for the rapid diagnosis of biological sex. In 1949, Dr. Murray L. Barr, a Canadian cytogeneticist was the first one to describe the phenomenon that in mammals, males are heterogametic (XY) and females homogametic (XX). In females, random inactivation of one of the two X chromosomes appears as darkly-staining bodies attached to the nuclear membrane called as Barr Body. It is a condensation of chromatin present at the nucleus of cells in female individuals and is a hetero-pyknotic, basophilic intra-nuclear structure demonstrable during interphase [4].

Various staining methods are used to demonstrate Barr Bodies like PAP stain, Leishman stain, Hematoxylin and Eosin (H & E), Thionine, Cresyl violet, Giemsa, and Aceto-Orcein[5].

Therefore, this study aimed to assess the effectiveness of buccal smear in gender determination and to compare the efficacy of the Giemsa and Feulgen stain for the detection of Barr bodies as compared to the routine stain.

MATERIALS AND METHODS:

This study was a cross-sectional descriptive study and it included 25 males and 25 females of age ranging from 5 years to 42 years who visited the Department of Oral and Maxillofacial Pathology at Government Dental College Aurangabad Maharashtra. Participants with tissue abuse habits (Tobacco, Gutkha, Areca nut chewing habits, Smoking habit, Alcohol habits) and oral lesions were excluded.

The subjects were properly explained the objectives of the intended study and consent was obtained. The subjects were asked to rinse their mouth with mouthwash and then with water. A sterilized disposable swab strip was scraped along the buccal mucosa of the cheek. The cellular material was quickly smeared on three clean slides. Three smears per participant were made and subjected to staining with H&E, Giemsa, and Feulgen stains. Smears were air-dried and placed in 95 % ethyl alcohol fixation for 30-35 minutes.
After fixation, all three smears were stained following the standard staining methods for Feulgen, Giemsa, and H&E stain.

Giemsa staining Procedure:

Fixed smears were stained with 10% Giemsa stain for 5-10 minutes. After staining, the slide was washed with distilled water to remove the excess stain.[2]Total time required was 15-20 mins.

Feulgen staining procedure:

After fixation smears were treated with graded alcohol (50% & 20%) in decreasing manner for 1 min each and then washed with distilled water. The slides were kept in 5 M hydrochloric acid for 30 mins and then washed under running tap water for 3 min. Further slides were drained and then treated with Schiff's reagent for 60 min. Then washed under running tap water for 5 min. After that slides were counterstained for 30 sec in 0.2% light green and rinsed well under running water for 2 min[6,7,8,9]. The total time required was 2hr.

Hematoxylin &Eosin staining procedure:

Fixed smears were treated with xylene for 10 mins followed by alcohol for 5 mins and with Hematoxylin stain for 5-6 mins. Then washed under running tap water for 3 min. The slides were given a dip in acid alcohol followed by 2-3 dips in ammonia and finally, stained with Eosin stain for 1-2 min. The slides were rinsed well under running water for 2 mins. The total time required was 25-30 mins[10].
The total 150 smears stained with 3 different stains were allowed to air-dry and then mounted with DPX to be observed under the light microscope.

The BB identification was determined by the presence of a darkly stained condensed area on the nucleoplasm.[11] The frequency of Barr body was examined by observing 100 nuclei per specimen under a binocular light microscope at 100 magnifications. The obtained values were tabulated and statistically analyzed for Kappa values and Students T test[12].

For all BB-positive cases Barr Body Index (BBI) was calculated using the following formula:

$$\frac{\text{Total number of BB counted cells} \times 100}{\text{Total number of cells (Approx.1000)}}$$

The categorization into males and females (gender determination) is based on a cut-off point (≤2: male, >2: female) as shown in table 2.

RESULTS:

After following the standard staining procedure, on examination Feulgen-stained smears showed nuclei stained with magenta color and cytoplasm green colored. Giemsa-stained smears showed nuclei stained violet, while the cytoplasm appeared light blue colored. H and E-stained smears showed nuclei blue and the cytoplasm pink.

The mean BBI values obtained for Feulgen, Giemsa & Routine stain were 34.30, 17.93, and 12.53 respectively (Table 1). The Feulgen stain scored better BBI with a mean value of 34.30% as compared to Giemsa with a mean BBI value of 17.93% and the Routine stain with a mean BBI value of 12.53%. When comparing the accuracy for identifying BB of all 3 stains Feulgen stain scored better BBI than Giemsa & routine stain.

The overall accuracy for BB identification using H and E, Giemsa and Feulgen stain was 94%,98%, and 100% respectively(Table no. 3). The Sensitivity of all 3 stained smears showed a 100% result. The specificity of the Feulgen stain, Giemsa stain, and Routine stain was 100%, 96%, and 94% respectively for detecting gender accurately. (Table no. 3) The gender determination based on BBI was considerably better with Feulgen stain than with Giemsa stain and routine stain in both males and females(Table no. 2).

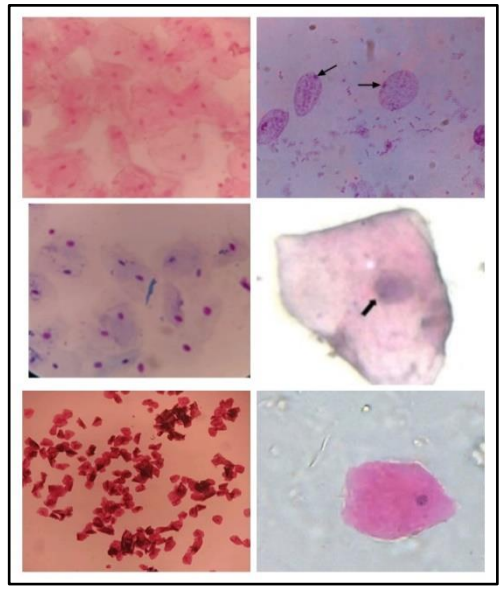

Fig.1 A) H&E-Stained Section Under 10x
B) Demonstration Of Bb By Using H&E Staining Technique Under 40 X
C) Giemsa-Stained Section Under 10x
D) Demonstration Of Bb By Using Giemsa Stain Under 40 X
E) Feulgen-Stained Section Under 10 X
F) Demonstration Of Bb By Using Feulgen Staining Technique Under 40x

Table no.1 to assess & compare BBI value among 3 different stain groups

	Female	***Male***
H&E Stain (Mean ± SD)	*12.528 ± 1.54*	*0.056 ± 0.14*
Giemsa Stain (Mean ± SD)	*17.932 ± 2.22*	*0.036 ± 0.081*
	34.3 ± 2.64	*0.024 ± 0.044*
F - Value	*674.96*	*0.71*
P-value	*<0.0000***	*0.496#*
F crit - Value	*3.12*	*3.12*

****Highly significant, #Non-significant**

Table no 2. Gender determination based on Barr body count using different staining methods

	H&E		**Giemsa**		**Feulgen**	
Barr bodies(BBI) [n(%)]	**Female [n(%)]**	**Male [n(%)]**	**Female [n(%)]**	**Male [n(%)]**	**Female [n(%)]**	**Male [n(%)]**
<= 2	**0(0%)**	**22(88%)**	**0(0%)**	**24(96%)**	**0(0%)**	**25(100%)**
>2	**25(100%)**	**3(12%)**	**25(100%)**	**1(4%)**	**25(100%)**	**0(0%)**

Table no 3. Sensitivity and specificity of study groups for sex determination

	H&E Stain	Giemsa Stain	Feulgen Stain
Sensitivity	**100%**	**100%**	**100%**
Specificity	**88%**	**96%**	**100%**
Accuracy	**94%**	**98%**	**100%**

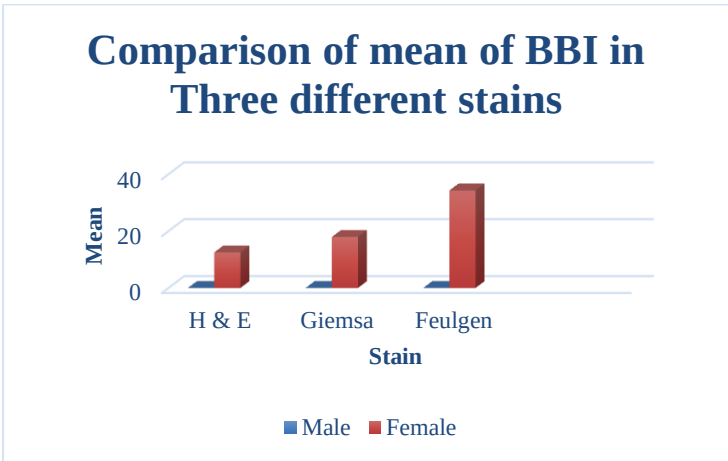

FIG.2 COMPARISON OF MEAN OF BBI IN THREE DIFFERENT STAINS

DISCUSSION:

Forensic odontology is an investigative aspect of dentistry that analyzes dental evidence for human identification. Apart from assisting in the identification of an individual, it reveals the age and gender of the same. Various methods have been used for the identification of sex. Sex determination can be done either by Morphological analysis (of the tooth, skull, and other soft tissues of the oral and para-oral region) or molecular analysis. In molecular analysis, BB plays a vital role in the identification of sex.

During embryonic development, females shut off one of their X chromosomes which undergoes random inactivation and condensation.[4] This inactivated X chromosome is fundamentally present in all the somatic cells of female mammals and is called as Barr body. It was first described by Barr and Bertram in 1949. It has a normal size of an average of 0.7–1.2 mm and is characterized as a round, oblong, triangular, plano-convex, or flattened body lying contiguous to the nuclear membrane internally[4]. The use of the buccal smear method for BB identification is widely popular as the specimens can be obtained with minimal inconvenience.[3,15]
Several stains have been used to demonstrate the presence of Barr bodies, including PAP, Aceto – orcein (AO), Feulgen, Guard, Cresol violet, Carbolfuchsin, and fluorescent staining methods. The Acridine orange is found to be ideal but not cost-effective. 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.

Taking these shortcomings, Feulgen and Giemsa stains are advantageous for their relative simplicity, and cost-effectiveness.[3,14] Giemsa stain is specific for the phosphate group of DNA. The Feulgen reaction is a stoichiometric procedure; each fixed molecule of Schiff's reagent corresponds to a constant and equivalent portion of the DNA molecule[7].

In the present study, the mean percentages of Barr bodies were consistently higher in females (100%) than in males (4-12%) which is in accordance with Tushar Mittal et al (2009),K.S Khanna et al study(2015), Talari Archana et al(2016), Kyaw Soe Htun(2017), Dr. Bahni Pathak et al (2022) [2,3,9,12,13].

In our study, H&E-stained smear showed a mean BBI of females 12.52±1.54 which is in favor of A Ravishankar et al 2018 (Mean BBI of females - 13 ± 2)[16]. Sensitivity was 100% which was similar and Specificity was found 88% which was concordance with KS Khanna et al 2015 (Sensitivity & Specificity - 100%)[9].
In the present study, Giemsa stained smear showed BBI of females and males was 17.932 ± 2.22 and 0.036 ± 0.081 respectively which was unfavored with Kyaw Soe et al 2017 (Female BBI-9.04 ± 3.583 and BBI Male 0.22 ± 0.616). Specificity was found similar to literature (Specificity- 98%) [3,10].

BBI was best identified under Feulgen stain. BBI of Feulgen stain in females and males was 34.3 ± 2.64 and 0.024 ± 0.044 respectively which is in accordance with KS Khanna et al 2015 (Female BBI-37.33±3.77, Males BBI-2.26±1.90).[9]

The sensitivity of all 3 stains was found to be 100%. The specificity of the Feulgen, Giemsa, and Routine stains showed 88%,96%and 100% respectively. The accuracy of the Feulgen stain, Giemsa stain, and H&E stain were 100%, 98%, and 94% respectively.

As Giemsa stain and Feulgen stain, both are cost-effective and less time-consuming methods for sex determination using BB but among them, the Feulgen staining method revealed a good diagnostic accuracy for gender determination. The visualization of BB with Feulgen is more than Giemsa and Routine stain. The fine nuclear details were effortlessly observed under the Feulgen staining method and Barr bodies were easily appreciated. So, the more preferable method for gender determination by measuring BBI is Feulgen stain.

CONCLUSION:

Feulgen stain had a better BBI and showed fine nuclear details compared to Giemsa stain and Routine stain. The statistical analysis showed 100% specificity, sensitivity, and accuracy in identifying Barr bodies. It is better advantageous than other staining method for rapid diagnosis, cost-effective & reliable with time.

CONSENT:

As per international standards or university standards, participant(s) written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL:

As per international standards or university standards are written ethical approval has been collected and preserved by the author(s).

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Conflicts of interest - There are no conflicts of interest

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