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COMPARATIVE STUDY OF EFFICACY OF PAPANICOLAOU AND ACETO-ORCEIN STAINS IN DEMONSTRATING BARR BODIES IN BUCCAL MUCOSAL SMEARS

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Abstract

Introduction: Establishing one's sexual identity has a critical role in medical conditions like ambiguous genitalia and in crime investigations. There are various investigations to determine the sexual identity of a person and Barr body evaluation in buccal smears forms the first line of investigation. Barr body estimation in buccal mucosal scrapes has been demonstrated by using different stains like Papanicolaou, Aceto-Orcein(AO), Feulgen, Guard, Cresyl violet, Carbol fuchsin and fluorescent staining methods, but with varying efficacy.

The current study is done to evaluate the efficacy of Papanicolaou and AO stains in demonstration of Barr bodies.

Materials and Methods: A total of 207 medical students were included in the study. Two buccal smears were collected from each student and subsequently, one was stained with Aceto-Orcein by squash technique and the other with Papanicolaou stain. Both slides were evaluated for percentage of Barr bodies using 1000x magnification and cytomorphological features in 400x.

Results: The percentage of Barr bodies in AO stained slides ranged from 5-18 among females and 0-8 in males, while with Papanicolaou stain the ranges recorded were 4 – 12 in females,

0 – 2 in males. The accuracy of AO and Papanicolaou stains for detecting sex accurately were 97% and 91% respectively. Evaluation of the buccal smears was better in AO stained smears because of the clean background and better cytoplasmic and nuclear contrast in comparison to PAP stain.

Conclusion: Aceto-Orcein staining method is rapid, economical, accurate, reproducible and comparable to Papanicolaou staining, for the detection of Barr body in buccal mucosal smears.

Keywords: Aceto-Orcein, Papanicolaou, Buccal smears, Barr body.

Introduction

Demonstration of genetical sex has an important role in sex determination. Sex identification can be done by various methods like morphometric analysis (of tooth, skull and other soft tissues of oral and perioral region) karyotyping, demonstration of fluorescent body (Y chromatin), polymerase chain reaction and Barr bodies. Among all the techniques, demonstration of Barr bodies is preferred, as it is done by simple staining methods.[1]

Most easily harvestable tissue sample for the Barr body evaluation is obtained from buccal mucosa, which can be performed by simple exfoliative cytology without inflicting trauma to the subject.[2]

Barr bodies develop as a result of inactivation of the X chromosome in normal female somatic cell by lyonization. They are Feulgen positive, heteropyknotic and basophilic intra nuclear structures demonstrable during interphase. All nuclear structures are known to fluoresce. Similarly, Barr bodies also fluoresce.[2,3]

Sex determination of a person can be done by demonstrating the percentage of Barr-body positive cells, as two non - overlapping ranges for the percentage of Barr-body positive cells have been obtained for men and women.[2]

Papanicolaou stain has been time tested and is in vogue even today for the estimation of Barr bodies in buccal smears. Even though it offers excellent contrast and is a simple procedure, it is time consuming, which has led to the increasing interest in utilizing stains like Leishman stain, Hematoxylin and Eosin (H & E), Thionine, Cresyl violet, Giemsa and Aceto-Orcein.

Amongst these staining methods being studied, Aceto-Orcein is rapid, chromatin specific and cost effective which is best suited for immediate screening of Barr bodies, in comparison to Papanicolaou stain. However, literature with regards to this comparison is scarce. So, to add to the available data, this study was taken up to assess the efficacy of sex determination by a comparison between Papanicolaou and Aceto-Orcein stains for the detection of Barr bodies in buccal mucosal scrapings.

Materials and Methods

Source of Data:

Apparently normal healthy students pursuing M.B.B.S in our institute formed the study population. A total of 207 students were included in the study and the study period was from 1st October 2016 to 30th June 2018.

Buccal mucosal smears were collected from the study subjects in the form of small groups ranging from 10-15 students at a time. Student was asked to rinse the mouth with water before the procedure. A wooden spatula was used to collect the material by drawing the spatula along the buccal surface of cheek. The collected cellular material was then transferred onto two clean glass slides and smears were prepared. The slides were immediately fixed using 95% ethyl alcohol.

Method of staining:

Wet fixed smears were divided into two groups. The study group smears were stained with Aceto-orcein and other control group smears were stained using Papanicolaou stain.

Aceto-Orcein staining Method:
Stock solution of Aceto-Orcein was prepared dissolving 1gm of orcein in 45ml of glacial acetic acid. The solution was boiled, cooled and filtered. The working solution was prepared by diluting 45 parts of stock solution in 55 parts of distilled water with periodic filtration.

Aceto-orcein Squash technique employed was as follows: One drop of working solution was placed in center of a smeared slide and a cover slip was placed over the drop. One or more layers of filter paper were placed over cover slip and firm pressure was exerted by drawing the thumb across the coverslip in a single direction, taking care to prevent the gliding of coverslip. The margins were sealed with DPX**.**

Papanicolaou staining method:

The slide was washed with water and then stained with Harris Hematoxylin for 5 minutes and washed with water, again the slide is dipped in 1% acid alcohol and blued for ten minutes, dehydrated in graded alcohols (75% , 95% and 95%) for two minutes each. After that stained in OG 6 for 2 minutes, then rinsed in 95% alcohol for 4 minutes and stained with EA 36 for three minutes and lastly rinsed in 95% alcohol for 1 minute and then slide was dried, cleared with xylene and mounted.

Smears with at least 100 cells with well preserved cytomorphological features were included in the study, whereas the smears with less than 100 cells were excluded (repeat collection and resmearing was done from those students) Under oil immersion, in 100 cells, the Barr body count was calculated in both the groups. ≤5% were recorded as male and those with >5% were recorded as female.
Criteria followed for Barr body Evaluation –

1. Cells with a fine vesicular or granular nucleus and well delineated nuclear border were considered.
2. To diagnose/ label as a Barr body, it should be present over the nuclear membrane, and appear in profile as a bar,or semidisc, or triangle, with the flat side against the nuclear membrane.
3. The length of the chromatin body should exceed 1 μm.
4. Cells contaminated with bacteria and cells with centrally located Barr-like bodies were excluded. Doubtful cells were considered as negative.

Results

A total of 207 students were included in the study group. Amongst which 97 (46.9%) were male and 110 (53.1%) were female. Mean age of females and males were 20.8 years and 21.1 years respectively. A total of 414 buccal smears were prepared from study subjects and stained with Aceto-Orcein and Papanicolaou staining methods as mentioned earlier. These smears were evaluated for the parameters like nuclear stain, cell morphology, background, overall staining and percentage of Barr bodies.
In case of Aceto-Orcein stained smears, crisp nuclear staining was noted in 206 smears whereas, 192 smears were found to have crisp nuclear staining in Papanicolaou method. Both the methods offered excellent nuclear staining in all the smears with absence of smudging of the nucleus.
Ninety nine percent of the AO smears had shown well preserved and crisp cell morphology. But in the case of PAP smears only 95.7% cases had well preserved and crisp cell morphology. These results were found to have a p value of 0.010 which was statistically significant. Both these techniques were capable of maintaining cell morphology, so they had not shown any unpreserved cell morphology.

Almost all cases of AO stained smears showed clean background. In comparison to AO stain nearly 10% of cases had shown bacilli in the PAP stain which was statistically significant with a p value <0.001.
Overall staining of both the stains were good. After considering parameters like nuclear, cytoplasmic and background staining characteristics, the overall staining was found to be comparable in both the groups.
After considering cut off of **Five** Bar bodies in hundred cells, the smears were categorized as males and females. As per the said criteria 96 were males and 111 were females in AO smears whereas in PAP stained smears, 115 and 92 were categorized as males and females respectively. However, these results were not statistically significant as the p value was 0.062.

Percentage of Barr bodies in two different groups

Comparing this data with the sex of the study subjects was done. The accuracy of AO smears was found to be 96.9% and 98.2% in case of males and females respectively. In case of PAP smears the comparison yielded accuracy rates of 90.7% and 92% in males and females respectively.
On an average mean Barr bodies were found to be higher in AO smears among both males and females when compared with PAP stained smears which was found to be statistically significant (p value < 0.001) (Table 1).
Mean percentage of Barr bodies, Percentage was almost double the number in the smears stained with Aceto-orcein (8.7) when compared with Papanicolaou stain (4.3) which

was found to be statistically significant with a p value <0.001. (Table 2).

The analysis of Sex determination by AO and PAP stained buccal smears has yielded a sensitivity and specificity of 98.18% and 96.91% whereas in Papanicolaou (PAP) sensitivity and specificity was 83.64% and 100% respectively. The accuracy of Aceto-Orcein and Papanicolaou in diagnostic efficacy in sex determination was 97.58% and 91.3% respectively. (Table 3).

Spearman's Correlation -

The Spearman's rank-order correlation is the non-parametric version of the Pearson product-moment correlation. Spearman's correlation coefficient, (ρ, also signified by r_s) measures the strength and direction of association between two ranked variables.

The spearman's correlation coefficient r_S is 1 for AO stain and it is 0.908 for Pap stain which is significant and the p value (<0.001) is significant for both the stains in calculating percentage of Barr bodies and identifying sex (Table 4).

Discussion

Even in the era of molecular testing, few tests have withstood the test of the time. One such test which is simple, rapid and repeatable with better accuracy is the buccal smear examination for sex determination which is important in cases of ambiguous genitalia, hermaphroditism, epicene and intersexuals. Further investigation of these cases forms the most common request for screening by buccal smear for Barr body evaluation.

Buccal mucosa forms an easy source of exfoliative cells with minimal invasiveness which can be performed even in field settings. The simplicity of this procedure will be of huge help in case of natural calamities, mass transit accidents, terrorist attacks and disasters.[4]

This procedure has undergone various modifications and improvements over the years. However, PAP stain is one of the preferred stain for Barr body analysis in buccal smears. Many authors have evaluated the utility of various other stains to reduce the time taken and to have better detection of Barr bodies with chromatin specific stains. Amongst these methodologies, Aceto-Orcein has both the advantages in the form of chromatin specificity and rapid staining in a matter of seconds further aiding in reducing the time taken.

In our study, among 207 apparently healthy individuals AO stained buccal smears had twice the detection capability of the Barr bodies with a mean Barr body percentage of 14.9% in females and 1.6% in males. Whereas, PAP stained smears were found to have a mean Barr body percentage of 7.5% in females and 0.5% in males. These results were statistically significant with a p value < 0.001. The better performance of AO smears could be due to the chromatin specificity of Aceto-Orcein and the absence of cytoplasmic staining which yielded a better contrast in evaluation of the nucleus.

Sex chromatin in male nuclei has been explained in oligophrenic subjects and various authors with different stains like Haematoxylin and Eosin, Feulgen and Acridine Orange to count the Barr bodies which were < 3% in male samples and samples like hair and dental pulp have also found Barr bodies in males ranging upto 8%.[5,6]

"The positivity for Barr bodies in males is due to the inheritance of males to carry primary sex organs of both the sexes. Y chromosome is one of the smallest chromosome and it varies in size from 6 million base pairs to 5 million, This is due to the repeated DNA pyro sequencing and gene silencing. Y chromosome contains genetic material for the development of male features and this development mainly occurs due to the Testis-determining factor (TDF). This factor is closely linked to a group of genes, called "sex determining region Y" (*SRY*) which is located on the short arm of the Y chromosome. The process of inactivation is a mystery, but it has been suggested that it is under the control of inactivation centre, located at Xq13. XIST, a gene which is transcribed from the inactive X, is necessary for initiation and propagation of X inactivation and does so by coating the inactive X. As inactive X is turned off by XIST allele, up to 21% of genes on Xp, and 3% on Xq may escape X inactivation" Which was proved by Lyon MF in human genetics in 1998.[7,8]

Using AO technique there was a better chance of detection of Barr bodies. These results were also noticed with the study done by Datar *et al* who have made a similar observation even by counting 50 cells for the calculation of mean Barr body percentage. In the present study, adopting the criteria for counting a minimum of 100 cells to arrive at the mean Barr body percentage has yielded a standard deviation of 2.2 and 1.6 in females and 1.1 and 0.6 in males showing an advantage over the counting of fifty cells as done by Datar et al study.[4] Detailed comparision tabulated in Table 5 (a) & (b).

To further evaluate the AO stained smears apart from analysing the mean Barr body percentage, cytomorphological features like nuclear staining , background, cell morphology and overall staining were also studied in both AO and PAP stained smears

The distinct advantage of AO smears over PAP stained smears was that the commensal organisms in the buccal mucosa were stained only in 1.5% of cases, which was 11% in the PAP stained smears. This enabled us in having better nuclear morphological appreciation with minimal overlapping and thereby reducing the screening time.

PAP, being the complex stain it is, with two counter stains for the cytoplasm had a clean background in case of 88.9% smears, whereas, AO smears had excellent contrast with clear demarcation of nucleus which enhanced the interpretation of buccal smears. In 99.5% of cases the absence of cytoplasmic staining in AO smears offered ease in counting the minimum number of cells required i.e., 100 cells, which was taken as a criteria in this study.

After considering the nuclear and cytoplasmic features in the form of overall staining both the stains had better morphology and were comparable. Based on these

observations buccal smear Barr body evaluation for sex determination has excellent sensitivity, specificity and accuracy which are essential for a screening test. Addition of Aceto-Orcein into the methodology retains this accuracy and reduces the overall analysis time, significantly. Cases which have been not identified conclusively by Barr body examination needs to be evaluated further by – blood samples for Davidson bodies, Karyotyping, FISH and Polymerase chain reaction.

Aceto-Orcein with its many advantages is an excellent choice. However the smears stained by following this procedure loses the staining characteristics over time. This is not an issue in case of analysis when rapid screening and reporting of results is done. Whereas, it will be a disadvantage if the smear has to be archived for review on a later date.

Barr bodies in buccal smears can also be determined with PAP stain , which has an equitable degree in determining sex chromatin. Because of its prolonged staining technique, cyto morphological features and various reagents Aceto-Orcein stood ahead in determining Barr body evaluation in clinical settings. Although, in few borderline subjects confirmatory tests like DNA profiling should be done, which is usually performed in subjects who lack the positive confirmation by gender determination as well as in discrepancy of genetic profile cases. Human dental pulp, which is an excellent source of DNA can be used for these purposes. One more advanced technique is with Amelogenin gene which can be done by pyrosequencing of short PCR products helps in conclusively identifying the genotypic gender of a person.

Conclusion

Sex determination can be done rapidly by determining the percentage of Barr bodies in buccal scrapes by Aceto-Orcein stain and PAP stain. Aceto-Orcein staining by squash technique is a rapid, economic and simple method providing 95-98% accuracy. It can be employed as a significant adjunct to other approaches of sex determination, even at a crime site. It can reduce staining time with accurate results, in rural areas and during natural calamities. It can be done with simple equipment and in a cost-effective way, whereas technically other methods of sex determination are not possible due to lack of sophisticated infrastructure in these settings. Yet, with all that said, in borderline cases, it is essential to utilize the more intricate methods of sex determination.

Table 1: Mean Barr bodies between study groups and sex

SMEAR	Barr bodies				p value
	MALE		FEMALE		
	Mean	SD	Mean	SD	
ACETO ORCEIN	1.6	1.1	14.9	2.2	<0.001*
PAPANICOLAOU	0.5	0.6	7.5	1.6	<0.001*

Table 2: Mean Barr bodies between study groups

PERCENTAGE OF BARR BODIES	ACETO ORCEIN		PAPANICOLAOU		p value
	Mean	SD	Mean	SD	
	8.7	6.9	4.3	3.7	<0.001*

Table 1: Efficacy of two staining methods

	ACETO ORCEIN	PAPANICOLAOU
Sensitivity	98.18%	83.64%
Specificity	96.91%	100.00%
PPV	97.30%	100.00%
NPV	97.92%	84.35%
Accuracy	97.58%	91.30%

Note: * significant at 5% level of significance (p<0.05)

Table 4: Spearman's correlation coefficient between sex and percentage of Barr bodies

Spearman's correlation coefficient	ACETO ORCEIN		PAPANICOLAOU	
	$r_s$value	p value	r_s value	p value
	1	<0.001*	0.908	<0.001*

Table 2(a) - Comparison of results of Aceto Orcein stain of present study with Datar et al [4]study

		Present study	Datar *et al.*[1]
Mean Percentage of Barr bodies	Females	14.9%	12.4%
	Males	1.6%	2.3%
Sensitivity		98.18%	98%
Specificity		96.91%	98%
Accuracy		97.58%	98.3%

Table 5(b): Comparison of statistical Parameters of Papanicolaou stain of present study with Datar et al [4]study

		Present study	Datar *et al.*[1]
Mean Percentage of Barr bodies	Females	7.5%	9.2%
	Males	0.5%	1.2%
Sensitivity		83.64%	90%
Specificity		100%	100%
Accuracy		91.30%	95%

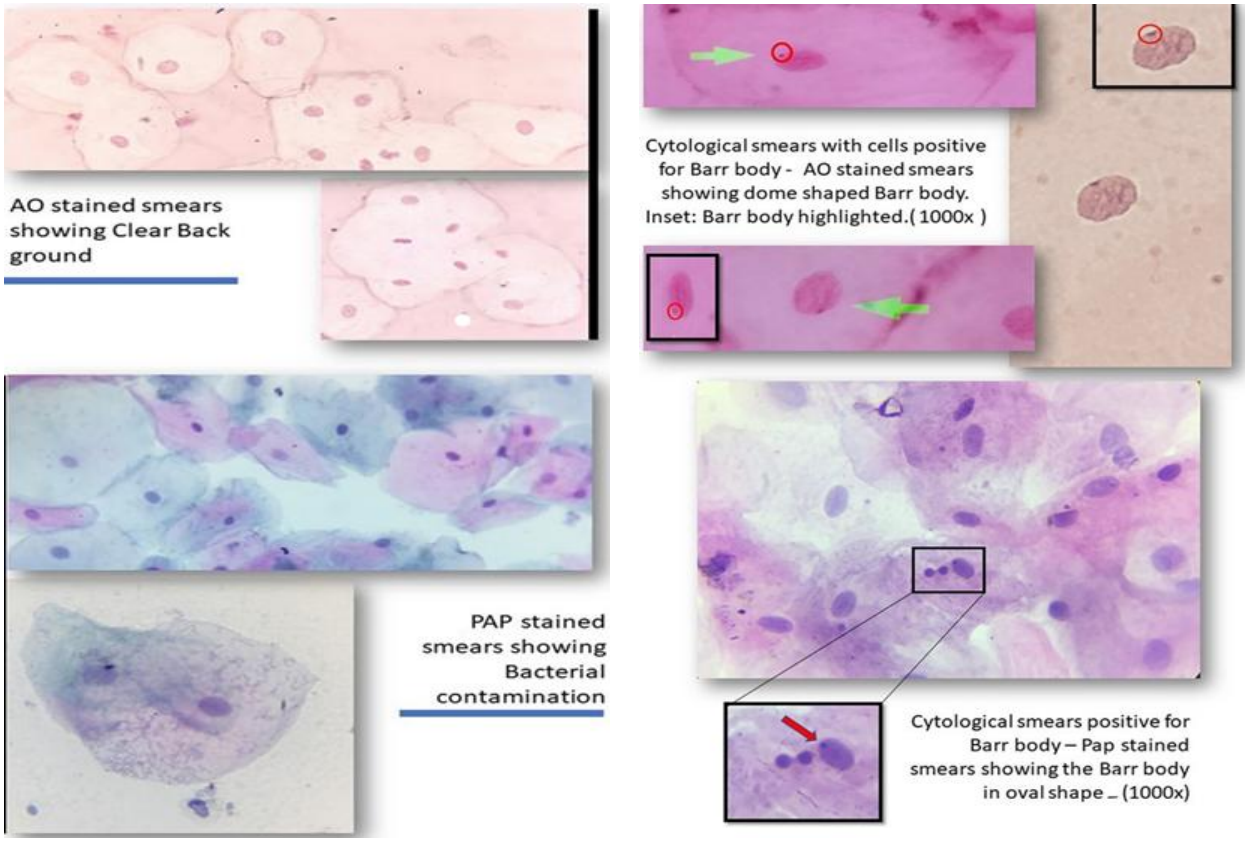

Figure 1: AO smears are showing clean background, PAP stained smears are showing Bacterial contamination.

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