ORIGINAL ARTICLE

Determination of sex by exfoliative cytology using acridine orange confocal microscopy: A short study

D Shyam Prasad Reddy, Herald J Sherlin[1], Pratibha Ramani[1], P Ajay Prakash

Department of Oral and Maxillofacial Pathology, Kamineni Institute of Dental Sciences, Narketpally, Nalgonda, Andhra Pradesh, [1]Saveetha Dental College, Saveetha University, Chennai, India

Address for correspondence:
Dr. D. Shyam Prasad Reddy, Department of Oral and Maxillofacial Pathology, Kamineni Institute of Dental Sciences, Narketpally, Nalgonda, Andhra Pradesh, India.
E-mail: shyamprasadreddy_d@yahoo.co.in

Abstract

Context: Establishing individuality is an imperative aspect in any investigation procedure. Sometimes, in identifying an individual, it becomes necessary to determine the sex of that particular individual. Combining rapidity with reliability, an innovative idea has been put forward using a confocal microscope in exfoliative cytology. In the present study, we have determined the sex of the individual from buccal mucosal scrapings. The exfoliative cells were observed for Barr bodies under a confocal microscope, and the percentage of Barr-body-positive cells was determined. **Aims:** The main objective of this study is to assess confocal microscopy for the determination of sex by observing Barr bodies in the exfoliative cells of both men and women. **Settings and Design:** Samples of buccal mucosa smears were made followed by acridine orange staining. The stained slides were observed under a confocal microscope and the data obtained was subjected for statistical analysis, especially for mean and standard deviation. **Materials and Methods:** Samples of buccal mucosa smears from 20 men and 20 women were obtained by scraping with flat wooden sticks (exfoliative cytology). The smears were fixed in 100% alcohol for 15 min, followed by acridine orange (AO) staining as described by Von Bertalanffy *et al*. Smears stained with AO were examined under a confocal microscope and the percentage of Barr-body-positive cells was determined. **Statistical Analysis Used:** Data obtained was subjected for statistical analysis, especially for mean and standard deviation. **Results:** Two non-overlapping ranges for the percentage of Barr-body-positive cells have been obtained for men and women. It was observed that in the male samples, the percentage of Barr-body-positive cells ranged from 0-3%. In the female samples, the percentage of Barr-body-positive cells ranged from 18-72%, and all the females showed the presence of Barr bodies. **Conclusion:** The study showed that the presence of Barr body in buccal mucosal cells can be demonstrated with a fair degree of accuracy using acridine orange confocal microscopy. The sex of the individual can be determined accurately with other advantages offered, such as the rapidity of processing and screening a specimen that results in saving of time.

Key words: Acridine orange staining, Barr body, confocal microscope, exfoliative cytology, sex determination

Access this article online	
Website: www.jfds.org	Quick Response Code
DOI: 10.4103/0975-1475.109887	

Introduction

Establishing individuality is an imperative aspect in any investigating procedure. There are numerous ways to do so in human beings (either alive or dead) when a human body is in its entirety but very few when only part(s) is available. Often, determination of the sex of an individual becomes important in situations like: for the purpose of

simple identification in the living, where the individual of one sex carries the features of the opposite sex; when a person appears to possess the primary sex organs of both the sexes; for the purpose of deciding whether an individual can exercise certain civil rights reserved for one sex only; for deciding questions relating to legitimacy, divorce, paternity, affiliation and also to some criminal offences; simple identification of dead individuals in an advanced state of decay where primary sex organs are lost due to decomposition.

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, fluorescent body (Y chromatin), polymerase chain reaction, Davidson body in the polymorphonuclear leukocytes, and Barr bodies (X chromatin).[1] The study of Barr bodies is advantageous in that it can be studied with simple staining techniques. The easily available material for Barr body studies is the buccal mucosa which can be obtained by performing simple exfoliative cytology without inflicting trauma on the subject.[2,3] Moore and Barr (1955) were the first scientists to introduce the buccal smear technique to identify sex.[4] The main aim and objective of the present study is to assess confocal microscopy for the detection of Barr-body-positive cells, thereby determining the sex which can be helpful in establishing the identity of an individual.

Materials and Methods

The present study has been reviewed by the institutional ethical committee and has therefore been performed in accordance with the ethical standards laid down in the 1965 Declaration of Helsinki. A total of 20 men and 20 women were selected randomly for the study after obtaining informed consent. Samples of buccal mucosa smears from 20 men and 20 women were obtained by scraping with flat wooden sticks (exfoliative cytology). The smears were fixed in 100% alcohol for 15 min, followed by acridine orange (AO) staining as described by Von Bertalanffy *et al*. The technique used for AO staining was as follows: the fixed smears were passed through descending grades of alcohol; then rinsed for a few seconds in 1% acetic acid and washed in two changes of distilled water for about 1 min; followed by staining in 0.01% AO for 3 min, de-staining in the phosphate buffer solution for 1 min; differentiated in 0.1M calcium chloride solution for 30 sec to 1 min (the nuclei should be clearly outlined). Excess calcium chloride was removed by washing with phosphate buffer solution and mounting was done with cover slip using a drop of phosphate buffer solution.[5] Smears stained with AO were examined under a confocal microscope.

The apparatus used in our study was Zeiss 510 LSM module Laser Scanning Confocal Microscope. The absorption range was 488 nm, blue, and the emission range was 505 nm, green. Smears stained with AO were screened using X10 and X20 objectives. Morphological detail of the exfoliative cells was studied with X40 objective and the microphotographs were taken.

One hundred cells were observed in each slide. Out of these 100 cells, the total number of Barr-body-positive cells (cells which showed the presence of a Barr body) was counted. As 100 cells were observed, this number became the percentage of Barr-body-positive cells. From the collected data the mean and standard deviation were determined.

Results

In the present study, acridine orange confocal microscopy showed the following results from the buccal smears of men and women. In the male samples, the percentage of Barr-body-positive cells ranged from 0-3% [Table 1]. Out of the 20 male samples observed, 18 showed the presence of Barr bodies [Figure 1]. Among these, eight had only 1% Barr-body-positive cells, while 10 had 2-3% Barr-body-positive cells. In the remaining two samples no Barr-body-positive cells (0%) were observed. None of the male samples had more than 3% Barr-body-positive cells.

In the female samples, the percentage of Barr-body-positive cells ranged from 18-72% [Table 1] and all the samples showed the presence of Barr bodies [Figure 2]. The majority of the samples (12 samples) had a lower percentage of

Table 1: Detection of Barr-body-positive cells in men and women

Sex	Range %	Mean	Standard deviation
Male	0-3	1.6	0.966
Female	18-72	47.1	18.929

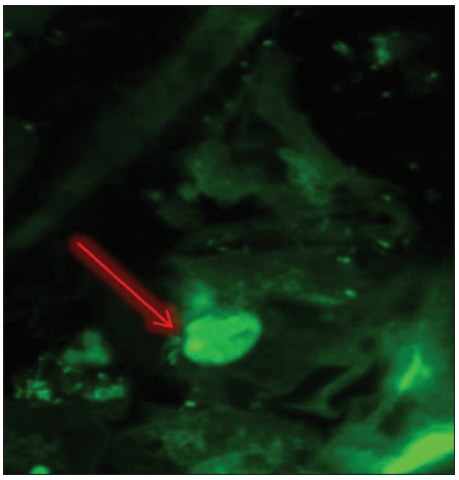

Figure 1: Acridine orange confocal microscopy showing Barr body (pointed arrow) in a male buccal smear (X40)

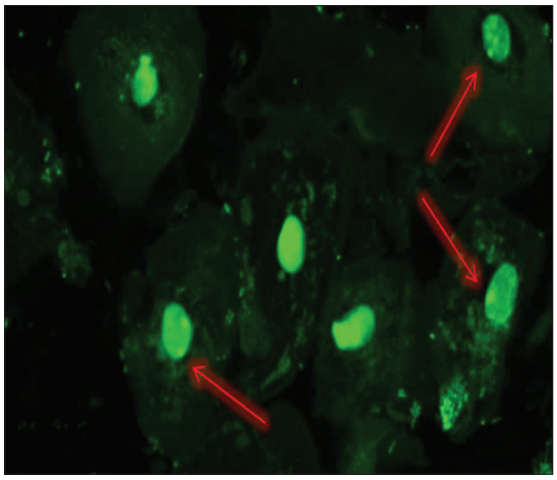

Figure 2: Acridine orange confocal microscopy showing Barr-body-positive cells (pointed arrows) in a female buccal smear (X40)

Barr-body-positive cells (18-50%) and in only a few (eight samples) it was greater than 50%. None of the women showed less than 18% Barr-body-positive cells.

Two non-overlapping ranges for the percentage of Barr-body-positive cells have been obtained for men and women.

Discussion

Determination of sex helps in criminal investigations for identification of the person, which can help in solving many cases of assault, theft, and sexual offences, etc. Human specimens, such as blood, semen, hair, and saliva stains containing buccal mucosal cells, found at the scene of crime or on a lethal weapon, are of major help in solving criminal cases. In cases of sexual offences, the buccal mucosal cells along with saliva stains are found in various parts of the body and also at the scene of crime. Saliva stains may be present on cigarette butts and also on cups and glasses which criminals have used. In train and aircraft accidents and also in natural disasters, it becomes difficult to identify the bodies. In such instances, buccal smears could help in detecting the sex and thereby establishing the identity.

The buccal smear technique to identify sex was first introduced by Moore and Barr in 1955.[4] The buccal smears were prepared and observed for the presence of Barr-body-positive cells which help in the determination of the sex. Barr bodies are named after the scientist Murray Barr who first described them.[4] 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. They can be plano-convex, biconvex, triangular, spherical, or rectangular in shape. Sometimes, they resemble the letter V, W, S, or X under an electron microscope. They measure about 0.8 to 1.1 μm in diameter.[2,3]

Barr bodies are known to arise from inactivation of the X chromosome in a female cell. This process of inactivation is known as lyonization, the process named after the scientist Lyon. In 1961, Lyon outlined the X-inactivation or what is commonly known as the Lyon hypothesis. It states that: Only one of the X chromosomes is genetically active; the other X of either maternal or paternal origin undergoes heteropyknosis and is rendered inactive; inactivation of either the maternal or paternal X occurs at random among all the cells of the blastocyst on or about the 16th day of embryonic life; and inactivation of the same X chromosome persists in all the cells derived from each precursor cell. Thus, the great preponderance of 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. Herein lies the explanation of why women have the same dosage of X-linked active genes as have men. The molecular basis of X inactivation is just beginning to be understood. It involves a unique gene called Xist, whose product is a non-coding RNA that is retained in the nucleus, where it "coats" the inactive X chromosome and initiates a gene-silencing process by chromatin modification and DNA methylation. The Xist allele is turned off in the active X.[6]

Confocal microscope, pioneered by Marvin Minsky in 1955, performs a point-by-point construction by focusing a point of light sequentially across the specimen and then collecting some of the returning rays. Currently, the confocal microscope is upgraded with a combination of lasers that can be coupled to the fiber optics of the scanning unit to increase the number of excitation wavelengths. Powerful software that displays and analyzes three-dimensional (3-D) data is added, making it a useful tool with the capability to quickly provide information about the biochemical and morphological details of the cells. The most important feature of the confocal microscope is the capability of isolating and collecting a plane of focus within a sample, thus eliminating the out-of-focus "haze" normally seen with a fluorescent sample. Fine detail is often obscured by the haze and cannot be detected in a non-confocal, fluorescent microscope. In the present study, confocal microscopy has been applied to cytology using acridine group of stains which is based on the affinity of the basic fluorochrome dyes for the nucleic acids.[7-12]

The present study was undertaken in search of a technique which combined rapidity with reliability. The results of the present study showed that it is possible to identify Barr bodies (Barr-body-positive cells) in the buccal mucosa, with a fair degree of accuracy, in both men and women by acridine orange confocal microscopy. The men in this study had 0-3% Barr bodies and women showed 18-72% Barr bodies in buccal mucosal cells. Strict criteria of selection for typical Barr bodies were taken into account without considering any influencing factors on the mean frequency.

For example, micronuclei were not considered because of their presence in the cytoplasm whereas Barr bodies were seen in the nucleus adjacent to the nuclear membrane. The added advantages of this technique are that high-resolution images are obtained, it has the ability to collect serial optical sections, and it is a valuable tool for 3-D reconstruction.[7-12]

In the present study, the percentage of Barr bodies in men could be compared to other similar studies with different staining methods. Manjula Bhai *et al*. did not report any Barr-body-positive cells in men.[13] However, there seemed to be a difference in the range and also the mean percent of Barr bodies among women in the present study as compared to other studies. A few studies reported a higher range and mean values,[14] whereas others found lower levels compared to the present study.[13] A comparison could not be made with some other studies because only the range and not the mean was available. While the present study had focused only on Indians, this study on Barr bodies had also been researched by other authors in different parts of the world. Interestingly, in one study done by Mittal *et al.*,[1] although the value of Barr bodies in men and women was different from the present study, the range and mean of Barr bodies among Indian men and women was comparable to the present study.

In many forensic cases, sex identification is absolutely essential. Considering the fact that the future of an individual is based on the reliability of tests such as amelogenin sex determination, DXYS156 tests etc,[15,16] we also suggest the inclusion of the study of Barr bodies (Barr-body-positive cells) in saliva for gender identification to further strengthen the evidence.

However, the Barr body test using AO confocal microscopy has a few limitations. Males with Klinefelter's syndrome tend to show one Barr body in each of their cells due to XXY and females with Turner's syndrome don't show any Barr body due to XO; in such cases the results may be wrong in identifying that particular individual where the test would identify the individuals as females for men with Klinefelter's syndrome, and women with Turner's syndrome would test as men.[17] Apart from these, a proper AO staining technique is required along with special fluorescent microscope equipment to visualize the stained smears. Prior experience is required to interpret the smears. Inexperienced technologists can misinterpret smears and sometimes may be overly sensitive. Last but not the least, the confocal microscope is expensive equipment when compared to the fluorescent microscope but has its own advantages as mentioned above.

Conclusion

Confocal microscopy was found to be reliable and accurate in the identification of Barr-body-positive cells in buccal mucosal scrapes. The sex of the individual could easily be identified by determining the percentage of Barr-body-positive cells, as two non-overlapping ranges for the percentage of Barr-body-positive cells had been obtained for men and women. The advantages offered by this technique are two-fold. First, the rapidity of processing and screening a specimen results in saving of time, attractive to a rapidly growing and increasingly busy department. Second, it adds a certain amount of objectivity to the process of establishing the identity of an individual. These considerations led to an attempt to assess the value of this method in determination of the sex that can be helpful in establishing individuality which is an imperative aspect in any investigating procedure.

References

1. Mittal T, Saralaya KM, Kuruvilla A, Achary C. Sex determination from buccal mucosa scrapes. Int J Legal Med 2009;123:437-40.
2. Hamerton JL. Human cytogeneties. Clinical Cytogenetics, Vol 2. New York: Academic Press; 1971.
3. Moore KL. The sex chromatin. Philadelphia: WB Saunders; 1966.
4. Anoop UR, Ramesh V, Balamurali PD, Nirima O, Premalatha B, Karthikshree VP. Role of Barr bodies obtained from oral smears in the determination of sex. Indian J Dent Res 2004;15:5-7.
5. Masin F, Masin M, Von Bertalanffy L. Use of acridine-orange fluorescence technique in exfoliative cytology. Science 1956;124:1024-5.
6. Plath K, Mlynarczyk Evans S, Nusinow DA, Panning B. Xist RNA and the mechanism of X chromosome inactivation. Annu Rev Genet 2002;36:233-78.
7. Singh A, Gopinathan KP. Confocal microscopy: A powerful technique for biological research. Curr Sci 1998;74:841-5.
8. Rowland RE, Nickless EM. Confocal microscopy opens the door to 3-Dimensional analysis of cells. Bioscience 2000;26:3-7.
9. Amos WB, White JG. How the confocal laser scanning microscope entered biological research. Biol Cell 2003;95:335-42.
10. Semwogerere D, Weeks ER. Confocal microscopy. Encyclopedia Biomat Biomed Engg 2005;10:1-10.
11. St Croix CM, Shand SH, Watkins SC. Watkins. Confocal microscopy: comparisons, applications, and problems. Biotechniques 2005;39(6 Suppl):S2-5.
12. Wanninger A. The application of confocal microscopy and 3D imaging software in Functional, Evolutionary, and Developmental Zoology: Reconstructing myo- and neurogenesis in space and time. Modern Research and Educational Topics in Microscopy. Badajoz, Spain: Formatex; 2007.
13. Manjula Bhai KH, Yadwad BS, Patil PV. A study of Barr bodies in Indian, Malaysian and Chinese subjects. J Forensic Med Toxicol 1997;14:9-13.
14. Aggarwal NK, Kumar S, Banerjee KK, Aggarwal BB. Sex determination from buccal mucosa. J Forensic Med Toxicol 1996;13:43-4.
15. Tschentscher F, Frey UH, Bajanowski T. Amelogenin sex determination by pyrosequencing of short PCR products. Int J Legal Med 2008;122:333-5.
16. Cali F, Forster P, Kersting C, Mirisola MG, D'Anna R, De Leo G. Romano V (2002) DXYS156: a multi-purpose short tandem repeat locus for determination of sex, paternal and maternal geographic origins and DNA fingerprinting. Int J Legal Med 2002;116:133-8.
17. Tucker R, Collins M. The science and management of sex verification in sport. SAJSM 2009;21:147-50.

How to cite this article: Reddy DS, Sherlin HJ, Ramani P, Prakash PA. Determination of sex by exfoliative cytology using acridine orange confocal microscopy: A short study. J Forensic Dent Sci 2012;4:66-9.

Source of Support: Nil, **Conflict of Interest:** None declared