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Comparative Durability of Common Stains Used for Exfoliative Vaginal Cytology

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ABSTRACT

In a study to compare the durability of commonly used stains (Giemsa, Leishman, Wright, Eosin, Nigrosin and Gentian violet) for exfoliative vaginal cytology, vaginal smear was obtained from eleven apparently healthy West African Dwarf (WAD) female Goats and processed according to standard technique. Scores (0-3) were given on four parameters namely background of smears, overall staining pattern, cytoplasmic staining and nuclear staining. Quality index one (QI-I) was calculated from the ratio of score achieved to the maximum score possible (12), immediately post staining while quality index–II (QI-II) was obtained 35 days after. Calculation for durability index (DI) was self-derived and equalled to ratio of QI-II to QI-I. The data were presented as mean ± SEM. Multinomial logistic regression model was generated for the QI-I and QI-II using durability index as reference category. Giemsa, Leishman and Wright stains were more durable than others with their mean DI values significantly ($P < 0.05$) higher than Gentian violet, Nigrosin and Eosin.The model showed 89.2 % overall model accuracy for the multinomial logistic regression model and 81.5% for the multinomial Bayes Naïve regression model. In conclusion, Giemsa, Leishman and Wright stains were more reliable and durable for exfoliative vaginal cytology compared to the other stains.

Keywords: Durability; Oestrus; Stains; Vaginal cytology

INTRODUCTION

Cytology as a diagnostic technique continues to enjoy wider acceptance and usage in clinical diagnosis because the technique is safe, quick, cost effective and accurate (Al-Abbadi, 2011). Meanwhile, cytology is delineated into two branches namely aspiration and exfoliative cytology. According to Durrant *et al.* (2003), Stockard and Papanicolaou were the first to recognise the specific diagnostic value of vaginal smear (exfoliative cytology) in guinea pig using the same Papanicolaou stain.

Exfoliative vaginal cytology (EVC) is a technique that has been used to characterise stages of oestrus in farm and domestic as well as laboratory animals. The technique is used in other farm/food animal such as goat (Ola *et al.,* 2006), cow (Miroud and Noakes, 1990), sheep (Rhendyka *et al.,* 2017), horse (Bader *et al.,* 1978) and laboratory animals like rat, mouse and rabbit (Cora *et al.,* 2015). Depending on the types of specificity of stains used, EVC is also used to diagnose pathological condition of the vagina such as malignancy (Fukushima *et al.,* 1986).

The exfoliated vaginal epithelial cells are categorised into parabasal, intermediate and superficial and their relative occurrence in presence or absence of blood cells like red blood cell, neutrophils vary with predominant hormone of reproductive tract and hence have been utilised to categorised oestrus stages (Wehrend *et al.,* 2013). The principle is based on the changes in proportion of these cells in response to predominant hormone either oestrogen or progesterone which vary during follicular or luteal phases of the oestrous cycle respectively (Montes and Luque, 1988).

Of all animal species, the method is well developed and widely maximised in canine because of increased in dog breeding activities and veterinarians been asked to provide services to determine the right time for breeding (Romagnoli, 2017; Arlt, 2018). In dogs, EVC is conventionally utilised for staging standing oestrus (Sharma and Sharma, 2016). It is also used to determine the time for optimal breeding (Raheem *et al.,* 2010). In WAD goat, EVC was reported to improve reproductive efficiency because of its ability to indicate accurately the time of oestrus/breeding post synchronisation (Leigh *et al.,* 2010).

After the first stain (Papanicolaou) for exfoliative cytology technique, several other stains have been used and subsequently optimised for EVC. These include Giemsa, Leishman, Wright, and Eosin stains. Others are Nigrosin and

Methylene blues. Ideally, a good stain must satisfy some conditions such as clarity, availability and durability. The clarity of the cytology smear depends on relative cell size, nuclear size, cytoplasmic details, smear background elements and visibility of intercellular matrix components. The stains must also be readily available and durable for a reasonable period before the smear stains on the slide are worn out and the cells therein start losing their characteristics. The vaginal smear stain is not expected to last indefinitely since it is not covered with a cover slip. However, it is good for the stain to retain its quality for a considerable length of time for at least a minimum of one month within which clinical diagnosis on the stained slide is expected to have been completed. Quality index is a conventional parameter used to compare different stains in cytological studies (Lanng *et al.,* 2019, Kamalkant Shastri and Joshi, 2020). Durability index derived in this study was used to compare the quality indices of the same stain obtained at two different times and served as a measure of resistance of the stain to change with time.

Sometimes the choice of best stain for EVC had been subject of controversy between the veterinary clinicians on duty. Therefore, this study was conducted with the objective of comparing the quality and durability of commonly used stains for EVC and to also provide an empirical evidence for sticking to a particular stain among the 6 stains for this procedure especially for clinical examination. Perhaps, the six stains used for the study are the most readily available in most laboratories and availability has been one of the major determining factors for choice of stain.

MATERIALS AND METHODS

Experimental Animals

Eleven West African Dwarf goats were obtained from Umudike, Abia State, Nigeria and surrounding areas. The animals were between 11 and 24 months of age with an average body weight of 14.2 ± 1.5 kg. The animals were accommodated in a concrete floor and roofed pen with individual animal staying in a room of about 1 mm^2 space. They were allowed to acclimatise to the environment for a period of one week before the commencement of the study. They were fed on lemon grass, cassava peelings, bean shaft and plantain. Water was made available *ad libitum*.

Experimental Design

One week post acclimatization, vaginal smears were prepared according to standard technique (Leigh *et al.,* 2010) and scored at the earliest possible time immediately post preparation. The slides were kept at room temperature (usually between 28-32°C) inside a slide box in one of the cupboards of Department of Theriogenology laboratory, College of Veterinary Medicine, Michael Okpara University of Agriculture, Umudike. A second scoring on the same slides was done five weeks later.

Vagina Cytology Technique

Six stains used in the present study were Giemsa, Leishman, Wright and Eosin stains. The other two were Nigrosin and Gentian Violet stains. Vaginal cytology was done according to standard technique as described in previous study (Leigh *et al.,* 2010). A sterile vagina swab was firstly soaked in normal saline briefly for 30 secs before insertion into the vagina to reach the anterior vagina through a 45^0 angulation into the vagina so as to avoid the vaginal fornix. The swab was made to touch the mucosa wall, after which it was gently drawn out to make impression (smear) on the slide. Six slides were produced this way and processed for the six different stains while the slides were appropriately labelled as per the stain and the animal number with a pencil on its edge such as LI-L11 for Leishman stain in goat number 1 to 11 and W1-W11 for Wright stains in goat number 1 to 11.

The slides were air dried for 3-5 mins before fixing the cells by immersing the slides in methanol for 15 mins after which the slides were gently rinsed in distilled water and stained with specific stains that include Giemsa, Leishman, Wright, Eosin, Nigrosin and Gentian Violet stains. Six slides were made from each animal and processed concurrently for all the stains using a slide rack since the only difference had to do with the final step of staining with a specific stain.

Microscopy

Immediately after processing the vaginal swab samples, the slides were then observed under microscope (Binocular Microscope DIDAC®, New Delhi, India) and images were taken at different objectives with the aid of AmScope System Microscopy (AmScope T49OPOT® Irvine, California, US). These images were stored for later evaluation and assessment to determine quality and durability indices.

Quality Index

Image quality was based on four parameters that include (i) background material staining, (ii) overall staining, (iii) cytoplasmic staining and (iv) nuclear staining. Each parameter had a score range of 0, 1, 2, and 3 in order of being poor, satisfactory, good and excellent respectively as was described in earlier study (Doddagowda *et al.,* 2017). The actual score (0-3) for each of the four categories of the cells aforementioned above made up the total actual score. The maximum possible for each parameter is 3 and a total of 12 for the four parameters such that the maximum possible for all the 11 animals for a particular stain is 132. The quality index one (QI-I) was calculated by dividing the total actual score obtained for each stain by the total maximum score possible (12). The slides were stored in a closed slide box (transparent top) and kept inside a cupboard within the laboratory. The scoring was repeated after 35 days of slide storage to get quality index two (QI-II). The final values of QI-I and QI-II were presented as mean + SEM for the eleven animals.

$$QI = \frac{\text{Total Actual Score (Background + Overall + Nuclear + cytoplasm)}}{\text{Maximum Score possible (3+3+3+3)}}$$

Durability Index

Durability index (DI) was self-derived taking a cue from the Quality index of day I and day 35 post staining and was a measure of the retainment of the image quality after a certain period of time and precisely 35 days in the present study. The DI was derived as the ratio of QI-II to QI-I. Therefore, the maximum DI was 1.0 in which the QI-II equalled QI-I. The

final values of DI for each of the six stains were presented as mean + SEM for the eleven animals.

Scoring of Staining

All the 65 slides used for this study were prepared and graded by one experience assessor to avoid variation in grading and the same protocol was followed. It is not impossible for each of the stains to require more or less time of incubation during processing, however the slides were processed in a similar manner with the same duration for smear air drying, fixing, rinsing and staining, all lasting a standard duration of 25 mins.

Statistical Analysis

The data were presented in form of mean ± standard error of mean and the effect of stains on quality index and durability indices were statistically analysed with Chi square. Pearson correlation was computed to check for multi-collinearity among the stains and Multinomial logistic and Naïve Bayes regression models were developed. Multinomial logistic regression model was generated for the QI-I and QI-II using DI as reference category.

Ethical Statement

The animals used followed humane treatment according to recommended protocol for use of animal for experimental research. Ethical approval was obtained from the research ethical committee of College of Veterinary Medicine Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria (MOUAU/CVM/REC/202021).

RESULTS

Oestrous Stages of the Entire Experimental Animals

The animals used in the present study were at different stages of oestrous cycle. Out of the eleven goats, 3 goats were in proestrus stage, while 4 were in oestrus. The number of goats in metoestrus and dioestrus were 2 each. Representative stained slides for Giemsa, Leishman and Wright stains are presented Figure I while that of Gentian violet, Nigrosin and Eosin stains are presented in Figure 2.

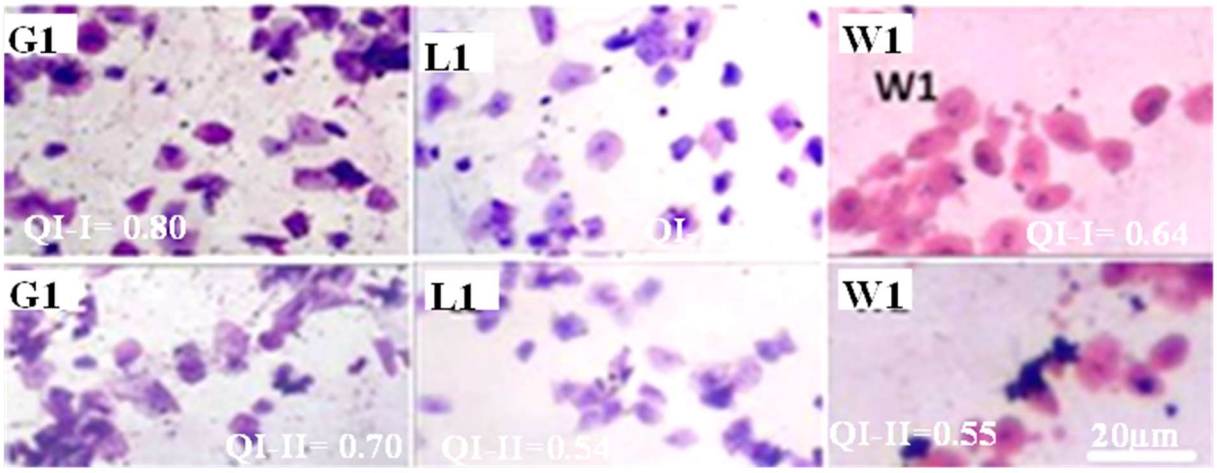

Figure 1: Representative Images of Stained slides used for quality index-I (QI-1) on day one of staining and for quality index–II (QI-II) at 35 days post staining (2). Legends: G- Giemsa, L; Leishman, W; Wright. The actual scores QI-I and QII are shown on the images.

Mean Quality Indices

Quality Index One –QI-I

The mean QI-I for all the stains across all the EVC for all the animals in the study ranged between 0.60 to 0.82 and these are presented in Figure 1. The highest mean QI-I was observed with Giemsa stain, (0.80 ± 0.02). This was followed by Nigrosin with mean QI-I values of 0.71 ± 0.04. The QI-I for Gentian violet and Leishman were 0.69 ± 0.03 and 0.67 ± 0.05 respectively. Eosin had QI-I values of 0.67 ± 0.03 while Wright stain had the minimum QI-I values (0.64 ± 0.04).

Quality Index Two- QI-II

The mean QI-II for all the stains across all the EVC for all the animals in the study ranged between 0.41 and 0.73 (Figure 1).

It was evidence that a reduction was observed of QI-I after 35 days of assessment during the 2nd assessment for all the stains used in the present study. The highest mean QI-II was observed with Giemsa stain (similar to QI-I evaluation), having mean QI-II values of 0.70 ± 0.03. This was followed by Wright (and not Nigrosin compared to QI) with QI-II values of 0.55 ± 0.04. The mean QI-II values for Leishman and Gentian violet were 0.54 ± 0.04 and 0.51 ± 0.04 respectively. Nigrosin had mean QI-I values of 0.48 ± 0.04 while Eosin stain had the minimum mean QI-II value which was 0.44 ± 0.03.

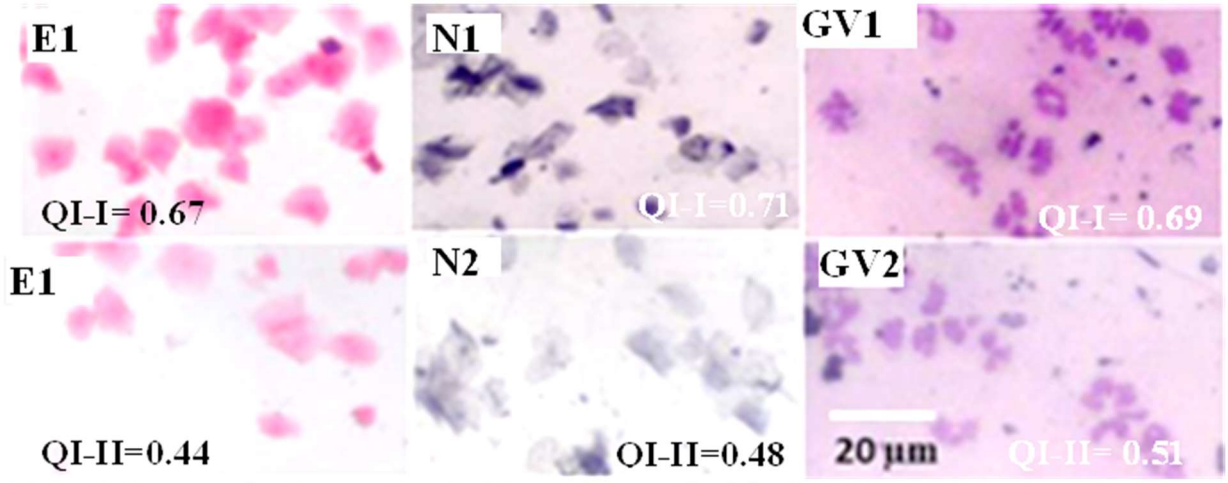

Figure 2. Representative Images of Stain slides used for quality index-I on day one of staining (1) and for quality index –II at 35 days post staining (2). Legends: E; Eosin, N; Nigrosin, GV; Gentian violet. The actual scores QI-I and QII are shown on the images

Mean Quality Indices
Quality Index One –QI-I

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Quality Index Two- QI-II

The mean QI-II for all the stains across all the EVC for all the animals in the study ranged between 0.41 and 0.73 (Figure 1). It was evidence that a reduction was observed of QI-I after 35 days of assessment during the 2nd assessment for all the stains used in the present study. The highest mean QI-II was observed with Giemsa stain (similar to QI-I evaluation), having mean QI-II values of 0.70 ± 0.03. This was followed by Wright (and not Nigrosin compared to QI) with QI-II values of 0.55 ± 0.04. The mean QI-II values for Leishman and Gentian violet were 0.54 ± 0.04 and 0.51 ± 0.04 respectively. Nigrosin had mean QI-I values of 0.48 ± 0.04 while Eosin stain had the minimum mean QI-II value which was 0.44 ± 0.03.

Mean Durability Index (DI)

The DI for all the stains across all the EVC for all the animals in the study ranged between 0.55 and 0.91 and are presented in figure III. The highest DI was observed with Giemsa stain (similar to QI-I evaluation), having mean values of 0.87 ± 0.03. This was followed by Leishman and Wright with mean DI values of 0.86. The DI for Leishman and Gentian violet were 0.79 ± 0.03 and 0.73 ± 0.03 respectively. Nigrosin had mean DI of 0.66 ± 0.30 while that of Eosin was 0.66 ± 0.03. There was a significant ($P < 0.05$) difference in the DI obtained for the six stained under this study.

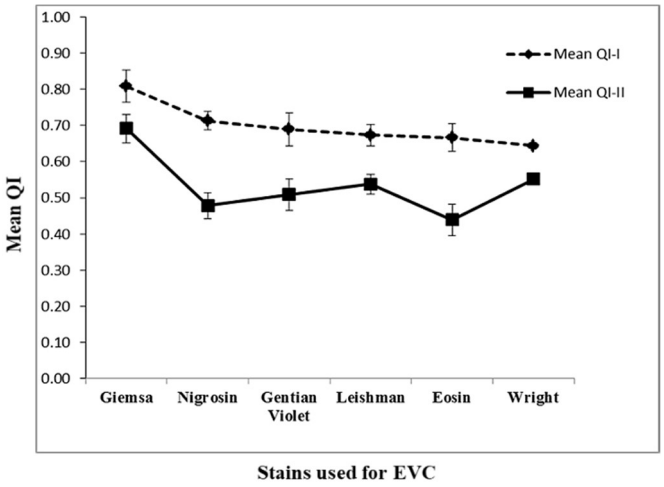

Figure 3: Line Graphs of Quality Index-I (QI-I) obtained on day I and Quality Index II (QI-II) obtained on day 35 post staining of the six stains (Mean ± SEM) from the eleven goats used for the exfoliative vaginal cytology (EVC) study.

Statistics with Naïve Bayes and Multinomial Regression

In this study, two different models were implemented into the frame-work and the results are presented in Table 1. Statistical analysis showed the sensitivity of the model, the specificity, posterior predicted value, Negative predicted value, prevalence, detection rate, detection prevalence and the balanced accuracy. Under the three indices considered namely QI-I, QI-II and DI, it was evident that Multinomial logistic regression produced better result in terms of model building than what Naïve Bayes produced. For the models, the accuracy rates for multinomial logistic regression and Multinomial Naïve Bayes were 95.0% and 81.9%, respectively for DI, 75.0% and 57.5% for QI-I respectively and lastly 86.36% and 76.39% for QI-II respectively.

Table 1: Statistics by Class for Naïve Bayes and Multinomial Regression

	Durability Index		**Quality Index I**		**Quality Index II**	
Model Statistics	Naïve Bayes	Multinomial Reg	Naïve Bayes	Multinomial Reg.	Naïve Bayes	Multinomial Reg
Sensitivity	0.7500	1.0000	0.4000	0.5000	0.7500	1.0000
Specificity	0.8889	0.9000	0.7500	1.0000	0.7778	0.7273
Pos Pred Value	0.7500	0.7500	0.5000	1.0000	0.6000	0.4000
Neg Pred Value	0.8889	1.0000	0.6667	0.5556	0.8750	1.0000
Prevalence	0.3077	0.2308	0.3846	0.6154	0.3077	0.1538
Detection Rate	0.2308	0.2308	0.1538	0.3077	0.2308	0.1538
Detection Prevalence	0.3077	0.3077	0.3077	0.3077	0.3846	0.3846
Balanced Accuracy	0.8194	0.9500	0.5750	0.7500	0.7639	0.8636

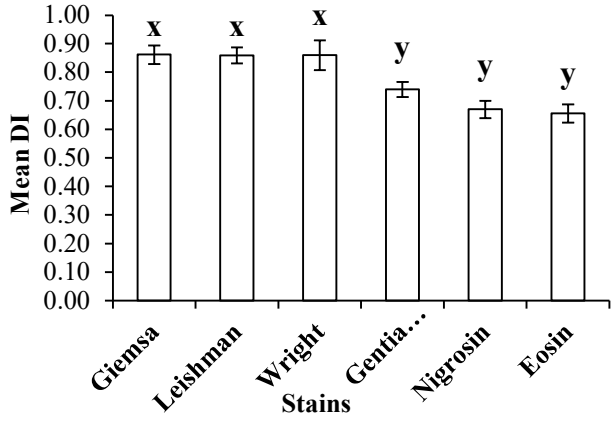

Figure 4: Bar charts of Mean Durability Index (DI) for the six stains (Mean ± SEM) from the eleven goats used for the study. Stains with no similar superscript are significantly different at $P \leq 0.05$. EVC; exfoliative vagina cytology.

DISCUSSION

The eleven animals used in the present studies were in different stages of oestrous cycle and that was normal since the goats were not synchronised prior to EVC and this was in agreement with previous report of variation of oestrous cycle among the same animal within a herd (Fatet *et al.,* 2011).

Perhaps, there was no ambiguity to recognise the stages of oestrous cycle with all the stains used in the study especially during the first evaluation which was done few hours post staining. For all the stains, there was a reduction of quality index at the second evaluation. The DI seems to follow the order of the QI-II. This is reasonable since the DI was a ratio of QI-II to QI-I. The more stable a stain was able to retain a value of QI-II very close to QI-I, the higher its DI and the less the changes observed in the values between quality indices done at the two time periods (i.e QI-I and QI-II).

A good stain must be clear enough to distinguish between different types of cells in the cytological field (Cora *et al.,* 2015). A major problem with some of these stains has to do with durability of the stained slide to allow for later viewing of the slides. This was peculiar with Nigrosin that was second to Giemsa stain for the QI-I values but came fifth for the QI-II and DI values. The durability of the stain slide is very essential for a number of reasons. The peculiarity of our environment as a less developed country is such that does not guarantee supply of electricity for research work at all or definite times. Therefore, prepared slides for EVC now may have to be kept and evaluated microscopically at a later time when the electricity becomes available. Besides, sometimes seeking a second opinion on the evaluated slides may also be necessary. Thirdly, the slides are also kept and used as teaching aid for clinical students of veterinary medicine/biomedical sciences. Henceforth, the durability of the stains is important if the morphology of the cells and the evaluation thereof are to be relatively stable for a reasonable period of time post preparation. Therefore, it is reasonable that the emphasis in the present study is laid more on durability of the stains rather than the immediate quality post staining (ie QI-I).

This study showed that amongst the stains used for EVC, Romanowsky-type stains (Giemsa, Wright and Leishman stains) having the highest mean QI-II as well DI values in that sequence. Giemsa produced the best results during the first and second evaluation and was adjudged the best durability among the six stains under the study with cytology similar to report of earlier studies (Leigh *et al.,* 2010, Raheem *et al.,* 2010, Leigh *et al.,* 2013). Beyond EVC, Giemsa stain is also currently used as the world's standard diagnostic technique for malaria's plasmodium, and it is also the basic stain for classifying lymphomas in the Kiel classification (Barcia, 2007). Generally, in the literature, Giemsa stain is one of the major stained used by veterinary practitioners for staging oestrus because of its ease to use and readily availability for haematology and other associate techniques in most laboratories.

Following Giemsa stain on DI were Leishman, Wright and Gentian violet while Eosin and Nigrosin stains had the least durability. The EVC with Giemsa and Leishman appeared similar to the reports of earlier investigator on this subject with epithelia cells having various shades of purple cytoplasm while the cell nuclei appeared deep blue and purple (Siregar *et al.,* 2016) whereas Wright-stained slides showed epithelial cells having violet-stained cytoplasm and the cell nuclei had various shades of deep blue and purple as have been reported in earlier studies (Leigh *et al.,* 2013).

Gentian violet, Nigrosin and Eosin had low durability and this is possibly responsible for non-usage for EVC in the literature. On the other hand, Eosin and Nigrosin are employed for liveability and morphological evaluations of spermatozoa (Bjorndahl *et al.,* 2003; Raheem *et al.,* 2009).

In comparison to Giemsa, Wright and Leishman stains, there is dearth of report on using Gentian Violet for vagina cytology; however, it was used in this study because it seems to be one of the most easily obtainable stain across the nook and cranny of the country. Gentian violet has antibacterial, antifungal, and anthelmintic properties and has been used as primarily antiseptic dye used to treat fungal infections of the skin such as ringworm athelete's foot with weak antibacterial effects and hence may be used on minor cuts and scrapes to prevent infection (Maley and Arbiser, 2013). The cytological features observed with GV in this study compared favourably with previous studies (Mclean *et al.,* 2012; Srinivasan *et al.,* 2017). The Gentian violet was reportedly used as adjunct for capsule visualization in cataract to visualise anterior lens epithelial cells (Andjelic *et al.,* 2014).

Sampling method is mainly dependent on the experience of pathologist and quality of staining depends on the type of stain and staining protocol followed (Almahmoud, 2009). As regards EVC, factors such as the collection site, the use of a speculum and the type of swab, intact or saline-moistened are predictive of the amount and intact morphology of vaginal cells obtained for rightful oestrous cycle staging.

Collection of vaginal cells is also possible without the use of a speculum as reported in earlier study (Aydin *et al.,* 2011). In some other studies, vaginal lavage method was used to facilitate the entrance of the swab for collection of adequate number of cells details (Srinivasan *et al.,* 2017). Whatever method of cells collection is chosen, the essential is to have enough cells free of contamination on the collection swab from the vagina and not the cervix or the vestibule for onward transfer on to the smear.

Vaginal cytology is mostly used in canine practice, however, its use in other farm animal especially goat is gradually gaining more ground having observed that evaluation of exfoliated vaginal cells at 24-hour interval has the potential to enhance detection of oestrus in synchronized West African Dwarf goat and subsequently increase reproductive efficiency (Leigh *et al.,* 2010).

Different procedure required different stains and staining technique; however, availability has been a major factor that drives the use of a particular stain and modification of a staining technique (Choudhary *et al.,* 2012). Apart from the six stains used in this study, other conventional cytological stains used for EVC include Papanicolaou (Pérez *et al.,* 2005), May-Grunwald, Boehringer Mannheim, Pappenheim and Testsimplets®, Harris-Schorr stain, modified Wright–Giemsa stain such as Diff-Quick® or Hemacolor®, methylene blue (Antonov, 2016). These stains are not readily obtainable around here. The choice of the six stains used was based on their availability within our laboratory. Vaginal smear may also be observed post fixation without staining (Aydin *et al.,* 2011) though the durability of the cell morphology is very limited.

The combination of two stains may produce better result. For instance, Leishma-Giemsa cocktail reportedly produced better results compared to May Grunwald Giemsa (MGG) for examination of air-dried fine needle aspiration cytology (Doddagowda *et al.,* 2017). A similar study to compare between Papanicolaou and crystal violet stains in vaginal cytology of rat preferred Papanicolaou to Gentian violet on the ground that the former produced a more detailed and clearer nuclear and cytoplasmic details (Srinivasan *et al.,* 2017). Such stains combination was not done in this study for simplicity of interpretation and hoping that this could be theme of subsequent further study on this subject.

Perhaps timing and cost of reagent were the main factors for revising Papanicolaou staining technique that has been used over the years for cervical cytology (Izhar *et al.,* 2014). In addition, the period of incubation of both slides and buffer solutions was demonstrated to have significant positive effect on the background, nuclear, cytoplasmic features and granules visualization of the stained slides on Leishman stain (Sareen *et al.,* 2018). However, these were not considered in the present study and could be subject matter of further research study on this theme since dealing with 6 stains at a time would make that endeavour too cumbersome and laborious.

The durability index was self-derived and the statistical analysis showed its balanced accuracy than either of QI-I or QI-II. The result of the multinomial logistic regression model showed 95.0% accuracy for Durability Index, 75.0% for Quality Index I and 86.4% for Quality Index II. This further gives credibility to our self-derived durability index over either of quality indices.

Conclusion

It is concluded that three out of the six stains used in this study, Giemsa, Leishman and Wright were quite durable after 35 days post processing. By implication, if they are properly kept as done in this study and further highlighted above, the slides may retain their good quality if not *ad infinitum*, definitely for a considerable long time. On the contrary, Gentian violet, Nigrosin and Eosin stains showed the least durability. Gentian violet is not a commonly used stain for EVC, however, with the results obtained in this study coupled with its readily availability, it is proposed to be a stain of choice especially in condition where Giemsa, Leishman and Wright are not easily obtainable, provided the slides are readable immediately post staining.

Conflict of Interest

The authors have no conflict of interest to declare.

Authors' Contribution

NVA and KAR were involved in conceptualization of the work, data collection and analysis as well as writing and approval of manuscript. TPO was involved in analysis and interpretation of data, writing and approval of manuscript. DD was involved in animal management, collection of data, writing and approval of manuscript. EOO and UIO participated in d writing and approval of manuscript.

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