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Research article

NONCONFORMITIES IN VETERINARY CYTOPATHOLOGICAL EXAMINATIONS: A RETROSPECTIVE STUDY OF UNSUITABLE SAMPLES FOR ANALYSIS

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The aim of this study was to evaluate the main nonconformities that result in cytopathological samples inappropriate for diagnosis in veterinary medicine. All cytopathological samples, obtained from different canine and feline tissues/lesions, included in the study were received and classified as inconclusive by a single public laboratory of veterinary pathology, located in Pernambuco State, Brazil, between 2012 and 2016. Nonconformities regarding the smear quality, cellularity, presence or absence of hemorrhage, cellular overlapping, desiccation, and presence or absence of necrotic debris and/or artifacts were evaluated. Data were tabulated using Microsoft Excel 2007; absolute and relative frequencies were calculated using EPIINFO 3.5.2. From the 3268 cases received between 2012 and 2016, 190 cases were selected and comprised 514 inconclusive slides. The most frequent nonconformities detected were insufficient/absence of cellularity in 100% (514/514), inadequate submacroscopic presentation in 87% (446/514), and hemorrhage in 69% (356/514) of samples. Other features identified were cellular overlapping in 34% (175/514), inadequate staining in 31% (175/514), artifacts in 30% (154/514), desiccation in 28% (145/514), and necrotic debris in 26% (133/514) of samples. The implementation of laboratory standard operational procedures aimed at maintaining quality is essential. It is necessary to initially identify the main errors occurring in the processing stages as a way to guide and design strategies to avoid them.

Key words: canine, cytopathology, feline, quality assurance, sample processing

INTRODUCTION

Cytopathological examination is a widely used technique in veterinary medicine characterized by low invasiveness, quick diagnosis, and low cost. Moreover, it allows

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the differential diagnosis of inflammatory processes from growth disorders, as well as the evaluation of the cellular types that compose a neoformation. This type of analysis is applicable for secretions, body cavity fluids, lavages, organs or solid tissues [1-4].

Nevertheless, careful interpretation is essential, since the technique does not allow any inference about the extension and depth of the lesion, and does not show the "architecture" of the tissue; in this way, with proper understanding of the technique's limitations, it can be sufficiently employed as a screening examination or, in some cases, as a definite diagnostic test [2-5].

Even though it is considered a simple procedure compared with histopathological analysis, cytopathological examination requires high responsibility during execution, since errors in this process may compromise the sample quality and final result. Detailed medical history, proper material quantity for lesion evaluation, adequate sample processing, and slide analysis by an experienced pathologist are factors that guarantee an appropriate diagnosis [6].

Failures in sampling and smear preparation may produce slides with low cellularity, contamination by blood and other artifacts, cellular overlapping, cell rupture, and staining issues [7-9]. The experience of the pathologist, along with precautions inherent to sample processing, are essential for the microscopic evaluation and correlation of these findings to the information provided about the patient [10].

This study aimed to evaluate the major nonconformities that produce cytopathological samples inappropriate for diagnosis in veterinary medicine.

MATERIALS AND METHODS

Case selection

This study covered all cytopathological examinations performed in a single public laboratory of veterinary pathology in Pernambuco State, Brazil, between 2012 and 2016. In this period, 3268 cytopathological examinations were performed, and 190 cases obtained from different tissues/lesions in canine and feline were considered inconclusive and resulted in 514 Diff-Quik-stained slides analyzed.

The proposed evaluation criteria referred to the sample collection technique as well as the evaluated tissue or organ, according to the information provided by the exam requisition form. The samples were obtained by fine needle aspiration, direct imprint and indirect imprint, however for some samples the collection technique was not specified in the request form.

Nonconformities evaluated

The selected slides were reevaluated by two experienced cytopathologists, considering two groups of nonconformities: macroscopic and microscopic. There was no disagreement among cytopathologists regarding the established nonconformities. The

macroscopic feature involved the submacroscopic evaluation, that is the smear quality (adequate or inadequate); the microscopic features were related to the cellularity (sufficient, insufficient and acellular), presence or absence of hemorrhage (>75% of the sample), cellular overlapping, desiccation, necrotic debris and artifacts such as glove powder and cotton fibers, and staining quality (adequate or inadequate).

Data analysis

The results obtained during the reevaluation of the slides were tabulated in spreadsheets using Microsoft Excel 2007 program and EPIINFO 3.5.2 was used to calculate the absolute and relative frequencies of each evaluated nonconformity.

Informed consent

The owners understood the procedures and agreed that the results related to the diagnostic investigation of their companion animals could be published in this journal.

RESULTS

The inconclusive cases obtained in this study corresponded to 5.81% (190/3268) of the total number of cases during that period. Regarding the sample collection technique, in 43.40% of the samples (223/514) the collection technique was not specified in the request form. Among the cases whose collection technique was specified in the request form, 55.44% of the samples (285/514) were obtained by fine needle aspiration (FNA), 0.58% (3/514) were obtained by direct imprint and 0.58% (3/514) were obtained by indirect imprint.

Regarding the organs and tissues analyzed through cytopathology, samples obtained from skin or subcutaneous tissue prevailed (Figure 1). The results of the evaluated nonconformities are displayed in Table 1.

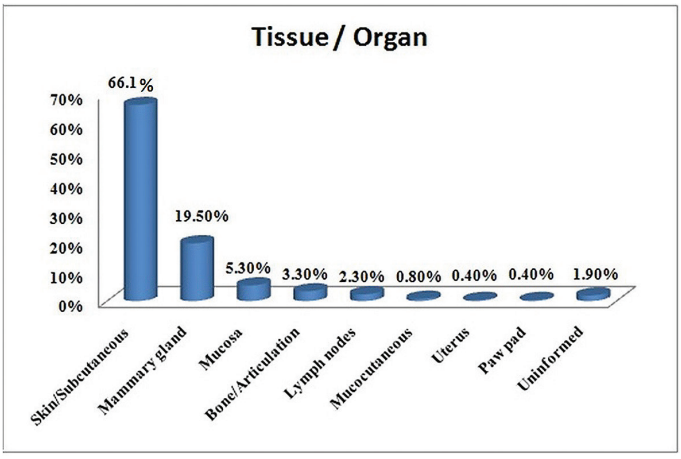

Figure 1. Relative frequencies of organs and tissues evaluated by cytopathological examinations of canines and felines, from 2012 to 2016.

Table 1. Main nonconformities observed in non-diagnostic slides, obtained from cytopathological examinations, performed from 2012 to 2016

	Nonconformity	Classification	AF	RF%
Macroscopic	Submacroscopic evaluation	Adequate	68	13%
		Inadequate*	446	87%
Microscopic	Desiccation	Present	234	46%
		Absent	145	28%
		Not applicable**	135	26%
	Staining	Adequate	219	43%
		Inadequate***	161	31%
		Not applicable**	134	26%
	Cellularity	Sufficient	0	-
		Insufficient	339	66%
		Acellular	175	34%
	Cellular overlapping	Present	175	34%
		Absent	204	40%
		Not applicable**	135	26%
	Hemorrhage	Present	356	69%
		Absent	158	31%
	Necrotic debris	Present	133	26%
		Absent	381	74%
	Artifacts	Present	154	30%
		Absent	360	70%
Total			514	100%

AF – Absolute frequency / RF – Relative frequency
* Inadequate: thick smear, sprayed material, material exceeding slide's borders, absence of material, and low cellularity.
**Not applicable: samples without cells and/or material, cytopathological evaluation not possible.
*** Inadequate: non-uniform staining, saturated areas and/or areas with little or no staining

The samples were grouped into two categories, considering the submacroscopic and the microscopic characteristics. Concerning submacroscopic characterization, five different nonconformities were identified in slides, individually or combined: smears exceeding the slide's borders, sprayed material, absence of material, thick smears, and continuity loss (Figure 2A-G).

From 446 inadequate slides in submacroscopic evaluation, it was verified that 73.1% (326/446) had only 1 nonconformity, 19.5% (87/446) had 2 nonconformities, 7.0% (31/446) had 3 nonconformities (Figure 2G) and 0.4% (2/446) had 4 nonconformities. Moreover, absence of material was observed in 37.9% (169/446) of the samples, thick smear in 29.8% (133/446), smears exceeding slide's borders in 32.7% (146/446), smear's continuity loss in 22.6% (101/446), and sprayed material in 11.7% (52/446).

Regarding microscopic analysis, slides characterized as submacroscopically adequate presented the following nonconformities: hemorrhage in 95.6% (65/68), low cellularity in 92.6% (63/68), and 7.3% of the samples (5/68) were characterized as acellular.

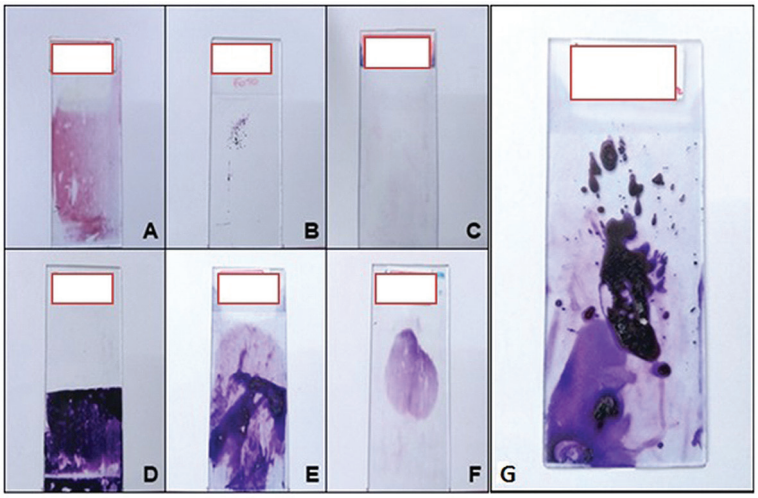

Figure 2. Submacroscopic features evidenced in cytopathological slides (Diff-Quik stain). **(A)** Material exceeding slide's borders, non-uniform and excessively pink stained; **(B)** sprayed material; **(C)** absence of material; **(D)** thick smear exceeding slide's borders with continuity loss; **(E)** thick smear exceeding slide's borders; **(F)** adequate smear, uniformly distributed material, uniform and balanced stain not exceeding the slide's borders; **(G)** combination of two or more nonconformities; thick sprayed smear that exceeds slide's borders.

By the same analysis, in slides submacroscopically characterized as inappropriate, low cellularity was observed in 61.9% (276/446), no cellularity in 38.1% (170/446), cellular overlapping in 37.7% (168/446) (Figure 3A-C), inadequate staining in 34% (153/446) (Figure 3B, C), desiccation in 31.4% (140/446) (Figure 3B, C), and hemorrhage (Figure 4A) was identified in 65.9% of the slides (294/446).

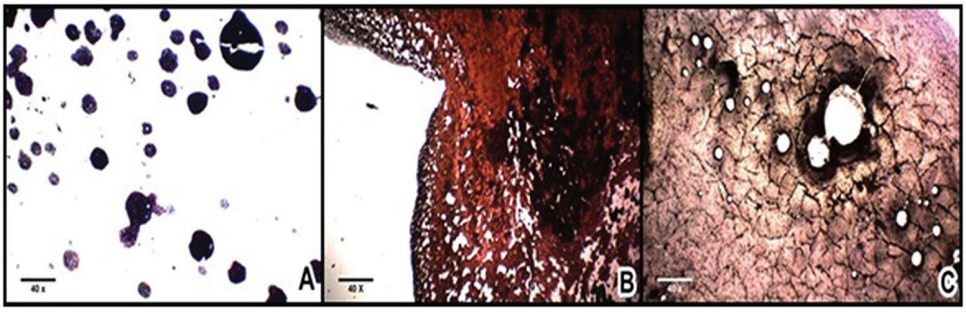

Figure 3. Microphotographs of cytopathological nonconformities. **(A)** Cytopathological sample with sprayed aspect; **(B)** thick cytopathological sample with visible overlapping of material, desiccation and inadequate staining; **(C)** thick cytopathological sample with desiccation, overlapping and non-uniform staining. (Diff-Quik, ×4 objective).

From the total number of slides, regardless of the submacroscopic evaluation, necrotic debris were evidenced in 26% (133/514) (Figure 4B) and contamination artifacts in 30% of the slides (154/514), with the main contaminants being glove powder (Figure 4C) and cotton fibers (Figure 4D).

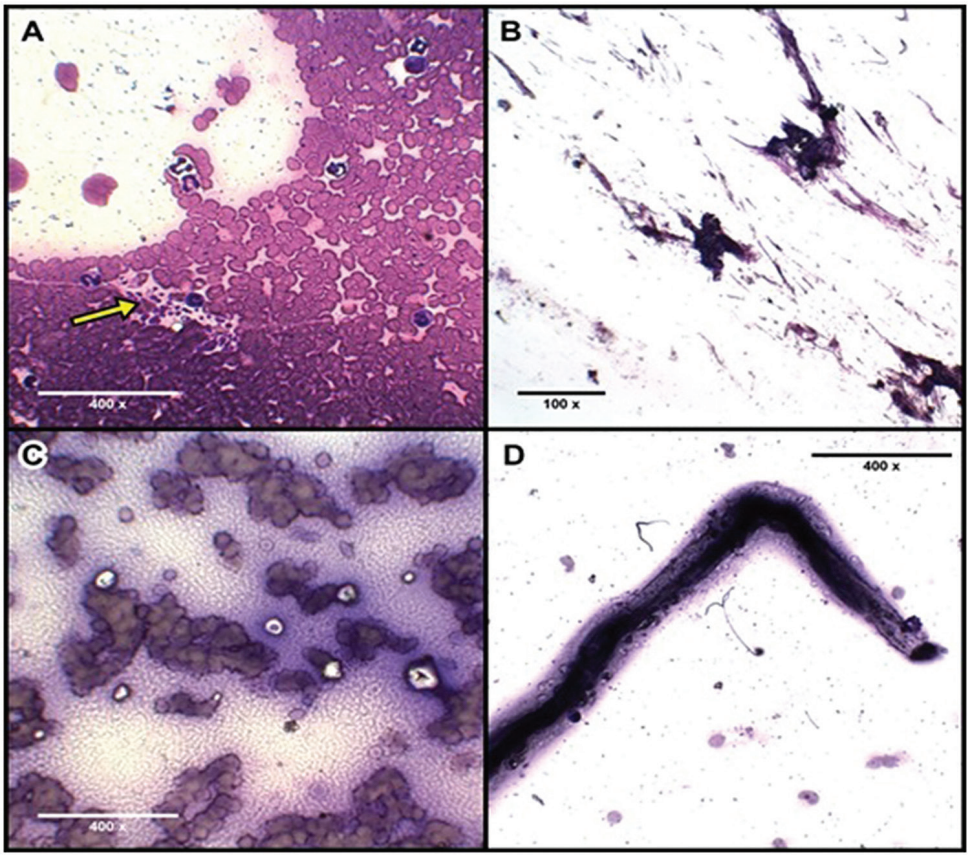

Figure 4. Microphotograph of nonconformities in cytopathological samples. **(A)** Hemorrhage with platelet aggregate (arrow, Diff-Quik, ×10 objective); **(B)** necrotic debris (Diff-Quik, ×10 objective); **(C)** contamination artifact introduced by glove powder (Diff-Quik, ×40 objective); **(D)** Contamination artifact introduced by cotton fiber (Diff-Quik, ×40 objective).

DISCUSSION

Diagnostic quality and proper laboratorial performance depend on variables related to pre-analytical, analytical, and post-analytical phases. Around 70% of the errors occur during the pre-analytical phase, which lasts from the exam request until sample collection and preparation and include exam requests non-concordant to the diagnosis, requisition forms with unreadable writing, absence of relevant clinical information and low sample quality [11].

This indicates the necessity for inherent responsibility of the professional who performs the sample collection, especially if it is going to be analyzed in a different laboratory by other professionals who have not examined the macroscopic lesion. Not only the collection technique, but also information such as the medical history,

progression period, and macroscopic description should be provided to guarantee the correct interpretation of the microscopic findings [9].

In this study, the total number of inconclusive cases corresponded to 5.81% of all cases examined, being proportionally classified as equal or lower when compared with the numbers of inconclusive cases reported by other authors [12-13].

Based on the obtained results regarding the sample collection techniques, FNA was frequently employed, due to its potential to be applied in a number of situations from superficial lesions, skin neoplasms, abdominal organs, mammary glands, and lymph nodes to harder tissues. Moreover, FNA allows the collection of samples of better quality because it avoids external contamination and allows the collection of cells from deeper layers [14-17]. This is important, since the sample may be acellular or present low cellularity due to inappropriate collection techniques. Thus, depending on the clinical suspicion and lesion type, the proper technique should be selected to minimize the risk of obtaining a non-diagnostic sample.

In this study, samples from skin and/or subcutaneous tissue prevailed. This tends to eliminate questions about the relationship between the collected tissue and presence of nonconformities or misdiagnosis. On the other hand, for cartilaginous and bone tissues there is a higher probability of obtaining samples with low cellularity or acellular samples due to low exfoliation. However, statistical evaluation was not employed to confirm this fact, because FNA surpassed other techniques by far.

Non-conforming submacroscopic features were identified in almost all slides and some samples exhibited more than one associated nonconformity, which could impair the diagnosis by material overlapping, desiccation, non-uniform staining, and other reasons. Errors in smearing occur during sample collection and are related with the technique employed [18]. Evaluating smearing quality allows the pathologist to identify possible artifacts and errors that might be present in microscopic evaluation. However, it is up to the professional responsible for sample collection and preparation, pathologist or not, to guarantee the maximum possible quality and improve the quality of the service provided by a cytopathology laboratory. The high turnover of employees during the evaluated period may justify collection techniques and smears with distinct aspects. Continuous education of the laboratory's team should be indispensable, focusing on diagnostic excellence.

Inadequate smearing generates microscopic nonconformities, such as the ones detected in this study. Slides without macroscopically visible material will probably not have viable material for a satisfactory microscopic analysis; results showed that slides submacroscopically classified as inadequate presented a higher frequency of acellular material (38.1%) than those classified as adequate (7.4%). Other submacroscopic features such as poor sample distribution on the slide, could produce thick smears and generate cellular overlapping, desiccation, and inadequate staining. This could be attributed to the fact that the usual fixation or staining time was insufficient because of the excess of agglomerated material [8].

Further, it was observed that some of the slides that presented hemorrhage also had insufficient cellularity. This can be justified by the hemodilution that results in the substitution of relevant cells in the delimited space of the blade. Even though it is possible to find a small amount of blood in some diagnostic samples, especially in particular types of lesion, the incorrect choice of sampling method may predispose for an excess of blood in the sample, increasing screening time and hindering microscopic interpretation [19-20].

All the evaluated slides had insufficient or no cellularity. The absence of sufficient material for analysis may occur due to inappropriate performance of the collection technique, for example, low pressure applied to aspirate firmer nodules, especially ones of mesenchymal origin, needle removal before plunger return, which results in sample suction into the syringe, besides the use of fine needle *non-aspiration* cytology for non-exfoliative lesions, that could limit the amount of collected cells [8,20,21].

Furthermore, desiccation, inadequate staining, and cellular overlap require attention, since although they are present in less than 50% of the samples they are evidenced with relatively high frequency and depend on the skills of the professional who performs the sample collections [8,18].

Staining errors are observed even in submacroscopic evaluation, as detected in this study, but can only be evidenced in microscopic analysis, in the form of saturated areas and/or areas with little or no staining, that could also be associated with desiccation. Several factors can lead to inadequate staining, all related to the preparation and maintenance of dyes and could be avoided by controlling these factors [8]. Frequent replacement and systematic filtration of the dyes favor proper staining and, thus, contribute to the diagnostic success. Other findings included necrotic debris and artifacts related to the sampling procedure. Debris can occur due to sampling performed in inappropriate sites of the lesion, or when exaggerated force is applied during smearing that results in the destruction of fragile cells [8,21].

Artifacts consist of artificial findings or structures accidentally introduced into the sample analyzed [22]. The artifacts found in this study were also produced during preparation and processing of the slides. Glove powder may resemble deposits of substances or microorganisms, while cotton fibers resemble fungal hyphae and filamentous pathogens [23,24]. Proper analysis of the lesion is essential in order to avoid sampling from unsuitable necrotic areas and prolonged exposure of the slides to the open air that could favor the deposition of suspended particles and artifacts.

The scarcity of studies that evaluate the quality of cytopathological analyses in veterinary medicine justifies the need for in-depth researches, aiming to improve the services provided to animals. Therefore, this is a pioneering study taking under consideration the difficulties faced in several veterinary cytopathology diagnostic laboratories in Brazil.

In conclusion, the detected nonconformities in veterinary cytopathological samples were originated from mistakes committed in the pre-analytical phase, involving both

sampling and slide processing. The implementation of laboratory standard operational procedures aiming to maintain quality becomes essential. It is necessary to first identify the major errors that occur in the processing stages as a way to guide and design strategies in order to avoid them.

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Authors' contributions

MBGS, ICP, and AAFO conceived of the presented idea. MBGS, ICP, OPS, ADFA, SRFG, MLMB, and MFP carried out the experiment. JWPJ performed the analytic calculations and helped supervise the project. AAFO accountable for all aspects of the work. All authors discussed the results and contributed to the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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NEUSKLAĐENOST CITOPATOLOŠKIH ISPITIVANJA U VETERINARSKOJ MEDICINI: RETROSPEKTIVNA STUDIJA NEPODESNIH UZORAKA ZA ANALIZU

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Cilj ove studije je bio da se procene glavne neusklađenosti koje rezultiraju činjenicom sa su uzorci za citopatološka ispitivanja neodgovarajući za postavljanje dijagnoze u veterinarskoj medicini. Svi uzorci citopatoloških analiza koji su uključeni u ovu studiju dobijeni su iz različitih tkiva/lezija od pasa i mačaka i kao takvi su klasifikovani kao neadekvatni od strane javne laboratorije za veterinarsku patologiju, locirane u Pernambuco (Brazil) u periodu od 2012. do 2016. Sa aspekta neusklađenosti kvaliteta razmaza posmatrani su parametri: celularna preklapanja, sušenje i prisustvo ili odsustvo nekrotičnog materijala i/ili artefakti. Podaci su tabelarno predstavljeni pomoću Microsoft Excel 2007, apsolutna i relativna učestalost je merena pomoću EPIINFO 3.5.2. Od 3268 uzorakaprimljenih u periodu od 2012. do 2016., 190 uzoraka (koje je činilo 514 preparata) bilo je neprihvatljivo. Najčešće neusaglašenosti su se ogledale u nedovoljnom broju ćelija (100%; 514/514), neodgovarajućoj submakroskopskoj prezentaciji (87%; 446/514) i u hemoragičnim uzorcima (69%; 356/514). Pored toga česta je bila pojava preklapanja ćelija (34%; 175/514), neodgovarajuće bojenje (31%; 175/514), artefakti (30%; 154/514), isušivanje (28%; 145/514) i nekrotični otpad u 26% (133/514) uzoraka. Implementacija laboratorijskih standarda tokom procedura je ključna za održavanje kvaliteta. Ključno je da se identifikuju glavne greške koje se dešavaju tokom procesa pripreme preparata, kako bi se njihovo ponavljanje kasnije izbeglo.