Inside the Cytologist - Training

- Highly interactive, +/- clinician-friendly environment
- Thorough & Descriptive
- Variably inhibited
  - Try not to over commit
  - Write a lot, particularly regarding rule outs
  - Don't be influenced by clinical information
  - May not always state what isn't there
  - Recommend biopsy

Inside the Cytologist – the Industry

- Case Load: 30-40 daily, up to 80.
- Reading & writing: 5-15 minutes per case
- Other Duties: Consultations, Path Reviews(!)
- Additional pressures: 2nd opinions, NC

Pragmatic Guide to Cytology Submissions

- Aspiration technique
- Slide preparation
- Slide numbers
- Pre-submission examination
- Slide labeling
- Request form
- **Get to know your Cytologist

Cytology Submissions: Summary

- Aspiration
  - Core spears ***vs*** Poke & Suck
  - Identify different intra-site aspirates
- Slide making
  - Evaluate pressure VS smear thickness
- Slide numbers
  - Strive for < 6 per site

Cytology Submissions: Summary

- Pre-submission examination
  - Just not the best ones
- Slide labeling
  - Patient id, site & intra-site, marking aids
- Request form
  - Lesion description & History
  - Always seen by the cytologist
  - Clinician queries: ***Encouraged***

Cytologic Expressions of Diagnostic Probability

- 2004 UCDavis Study
- 96 ACVP responders (60% academic)
- Evaluated 18 modifiers/terms
  - Frequency of use
  - Preference based on level of DX probability
  - Factors that affect diagnostic certainty
  - Examined experience, employment, gender

Cytologic Expressions of Diagnostic Probability

- "Diagnostic for..
- "Most consistent with..
- "Probable..
- "Possible..
- "Unlikely..
- "Highly suggestive of..
- "Consistent with..
- "Suggestive of..
- "Cannot rule-out..
- "No evidence for..

Expressions of Diagnostic Probability - % Certainty

- No modifier: 100% (95)
- "Diagnostic for: 100% (95)
- "Highly suggestive of: 95% (75)
- "Most consistent with: 95% (50-75)
- "Consistent with: 75-95% (50-100)
- "Probable: 75-95% (50)
- "Suggestive of: 50-75% (25)
- "Possible: 25-50% (5)
- "Can't rule out: 5-25% (50)
- "Unlikely: 5-25%
- "No evidence of: 0-5%

Cytologic Expressions of Diagnostic Probability: Conclusions

- Expectedly rich and individualized lexicon
- Sample quality and clinical information influence relative certainty
- Experience, commercial lab employment increase certainty implied by terms
  - Higher probability of diagnosis with less definitive modifiers
- Disconnect remains between the terms and the clinician's interpretation

Cytologic Expressions of Diagnostic Probability

- "Because of wide discrepancy in the implied likelihood of a diagnosis using ***words***, defined terminology may be useful in improving the quality of data in cytology reporting" *Christopher & Hotz, 2004*
- Medicine is a science of uncertainty and an art of probability – ***Osler***

Clinician Interpretation of Cytologic Expressions: Summary

TERM	***Pathologist***	***Clinician***
• Diagnostic:	100%	99%
• Highly suggestive:	90%	75%
• Most consistent:	90%	80%
• Consistent*	85%	70%
• Probable*	80%	66%
• Suggestive*	75%	60%
• Possible*	50%	50%
• Can't rule out:	15%	30%
• No evidence of:	0-5%	0-25%

Clinician Interpretation of Cytologic Expressions: Summary

- Cytologist's terms tend to understate their estimation of disease probability.
- Clinicians may be confused by these terms and, comparatively, understate their interpretation of disease probability.
- Clinicians, but not cytologists, prefer a %.
- Based on these expressions, treatment actions may be relatively conservation.

Clinician Interpretation of Cytologic Expressions: Disease Management

- ***Consistent with*** Lymphoma
  - Therapy vs Follow-up: 50/50
  - Euthanasia: 62%
- ***Probable*** Lymphoma
  - Therapy vs Follow-up: 15/85
  - Euthanasia: 35%
- ***Suggestive of*** Lymphoma
  - Therapy vs Follow-up: 4/96
  - Euthanasia: 8%
- ***Possible*** Lymphoma
  - Therapy vs Follow-up: 2/98
  - Euthanasia: 2%

Cytology versus Biopsy: Comparisons of Accuracy

Cytology versus Biopsy: Comparisons of Accuracy

- Issues and Protocols

- Many Investigations - by tissues and sites

Bony Lesions	Respiratory Tract
GI Tumors	Cutaneous & Subcutaneous
Mammary Tumors	Liver Lesions
Splenic Aspirates	Lymph Nodes
Prostatic Disease	Body Cavity Effusions

Widely Variable Accuracies Between Studies

Cytology versus Biopsy: Protocol for Validation and Test Comparison

- STARDS Guidelines
  - Gold Standard compared to new test
  - **TBA: Other requirements
- Defines test specificity, sensitivity, etc.
- Basis for determination of accuracy for all new tests (including cytology)

Cytology versus Biopsy: Protocol and Variables

- Patient with biopsy preceded by recent cytology of same tissue.
- Variables:
  - Timing between sampling
  - Proximity of tissue specimens
  - Relative accuracy of different forms of biopsy
  - Report review vs re-evaluation of slides
  - Discrepant definitions of diagnostic 'agreement'

Cytology versus Biopsy: Definitions of Accuracy

- ***Diagnostic sensitivity*** **- the frequency a test is positive in patients that have the disease of interest; a test that has a high sensitivity has *few false negative* results and is suitable in *screening* for the presence of the disease.**
- ***Diagnostic specificity*** **- the frequency a test is negative in individuals that do not have the disease of interest; a test that has a high specificity shows *few false positives* and, thus, is suitable for *confirming* the presence of the disease**

Cytology versus Biopsy: Definitions of Accuracy

- ***Diagnostic accuracy*** **is the frequency a test correctly identifies a patient as having, or not having, the disease of interest; a test with high diagnostic accuracy reveals relatively few false positive & false negative results.**
- **NOTE: Cytology is often performed to identify more than one disease process. Thus, accuracy can also be defined based on how often it agrees (correlates) with the gold standard - biopsy.**

Cytology versus Biopsy: Tissue 1

- Study Specifics
- Data
- Special Comments

Cytology versus Biopsy: Tissue 2

- Study Specifics
- Data
- Special Comments

Cytology versus Biopsy: Tissue 3

- Study Specifics
- Data
- Special Comments

Cytology versus Biopsy: Tissue 4

- Study Specifics
- Data
- Special Comments

Cytology versus Biopsy: Tissue 5

- Study Specifics
- Data
- Special Comments

Cytology versus Biopsy: Tissue 6

- Study Specifics
- Data
- Special Comments

Cytology versus Biopsy: Summary

Molecular Diagnostics

- Flow Cytometry
- Clonality: PCR for Antigen Receptor Rearrangement (PARR) Assay
- Immunohistochemistry (IHC) and immunocytochemistry (ICC)
- C-kit analysis via PCR for MCT
- Genetic sequencing

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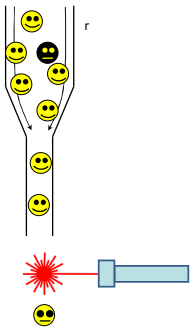
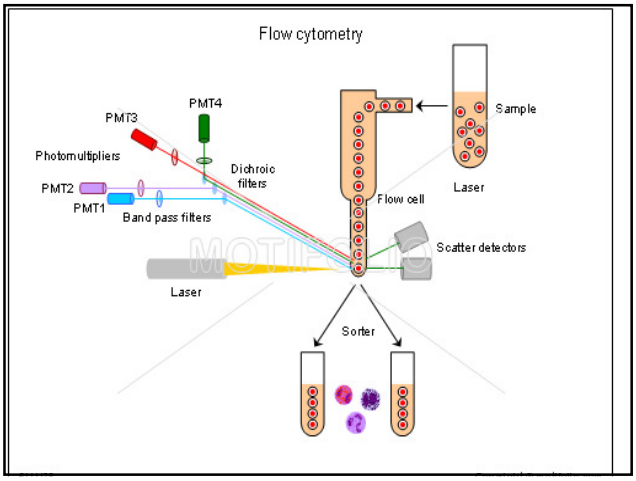
Flow Cytometry

-Requires cells within a fluid to be directed, single-file, into the path of a laser.

-Deflection and scatter of the beam demonstrates unique characteristics of each cell.

- Use of fluorescent markers creates additional data that identifies other features, including CD proteins for immunophenotyping.



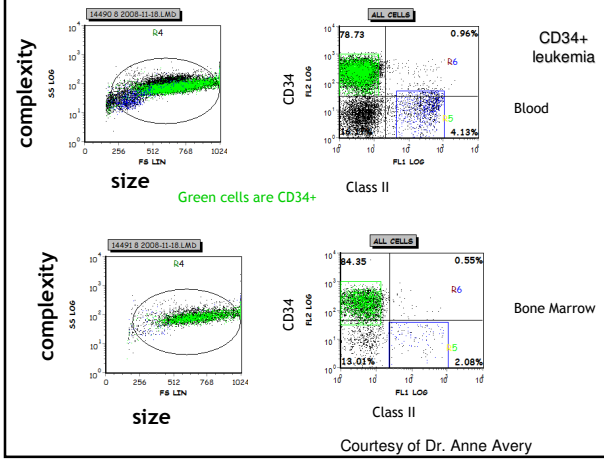


Flow Cytometry: Specimen Requirements

- Viable cells (50-200K) in fluid
- Whole blood, marrow (LTT – EDTA)
- Body cavity effusions
- Tissue aspirates into Saline:Serum (1:9)
- Keep chilled, ship overnite, weekday arrival

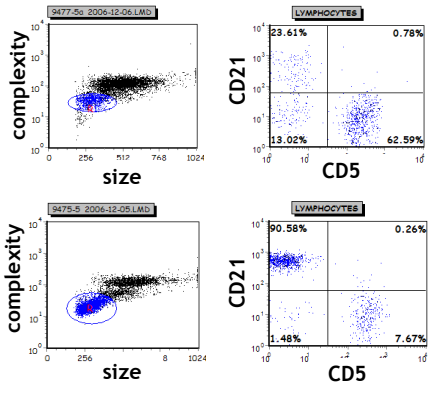
Flow Cytometry - Indications

- Differentiates between neoplastic and immunostimulated lymphocytes
- Lymphoproliferative Disease: Immunophenotype (T- versus B-cell)
- Provides detailed phenotype subset information – including aberrent forms
- Distinguishes stem (primitive) cell from more differentiated lymphocyte population
- Prognostic value: T zone lymphoma vs CD4+ T cell lymphoma

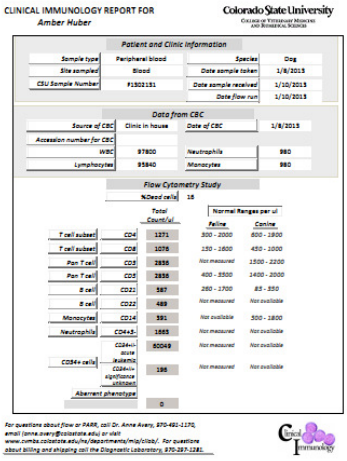


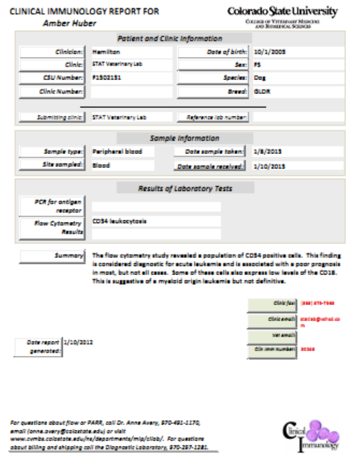
Courtesy of Dr. Anne Avery

Homogeneous expansion of B cells in the peripheral blood



Courtesy of Dr. Anne Avery





CLINICAL IMMUNOLOGY REPORT FOR
Amber Huber

Colorado State University
College of Veterinary Medicine and Biomedical Sciences

Patient and Clinic Information

Clinician:	Hamilton	*Date of birth:*	10/1/2003
Clinic:	STAT Veterinary Lab	*Sex:*	FS
CSU Number:	F1302151	*Species:*	Dog
Clinic Number:		*Breed:*	GLDR
Submitting clinic:	STAT Veterinary Lab	*Reference lab number:*	

Sample Information

Sample type:	Peripheral blood	*Date sample taken:*	1/8/2013
Site sampled:	Blood	*Date sample received:*	1/10/2013

Results of Laboratory Tests

PCR for antigen receptor	
Flow Cytometry Results	CD34 leukocytosis

Summary The flow cytometry study revealed a population of CD34 positive cells. This finding is considered diagnostic for acute leukemia and is associated with a poor prognosis in most, but not all cases. Some of these cells also express low levels of the CD18. This is suggestive of a myeloid origin leukemia but not definitive.

Clinic fax: [illegible]
Clinic email: [illegible]
Vet email:
Clin imm number: [illegible]

Date report generated: 1/10/2013

For questions about flow or PARR, call Dr. Anne Avery, 970-491-1170, email (anne.avery@colostate.edu) or visit www.cvmbs.colostate.edu/ns/departments/mip/cilab/. For questions about billing and shipping call the Diagnostic Laboratory, 970-297-1281.

Clinical Immunology

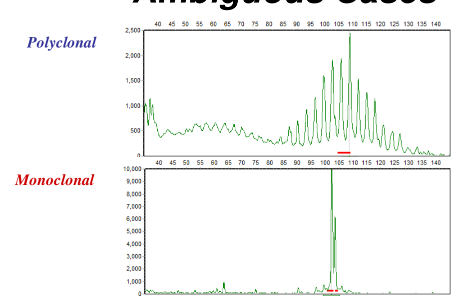
Clonality (PARR) Assays: Methodology

- Determines if a lymphoid population is derived from a single (neoplastic) clone.
- Uses PCR to identify a variety of immunoglobulin receptor genes (B-cells) and T-cell receptor genes.
- Single-sized PCR product, versus an admixture (polyclonal) distinguishes clonality from lymphoid hyperplasia.

Clonality (PARR): Indications

- Differentiates between lymphoid elements in non-neoplastic inflammation versus lymphoproliferative conditions.
- Helpful in cytologically ambiguous lymphoid hyperplasia/dysplasia
- High detection level - 1:100 tumor: normal cells, yet poor sensitivity (70%)
- Less sensitive/specific compared to Flow

Using Clonality to Resolve Ambiguous Cases

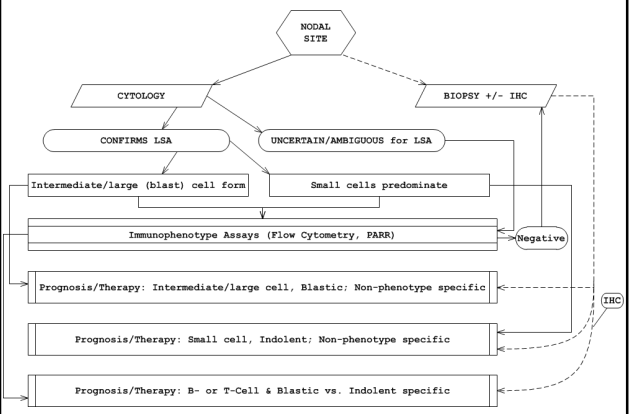


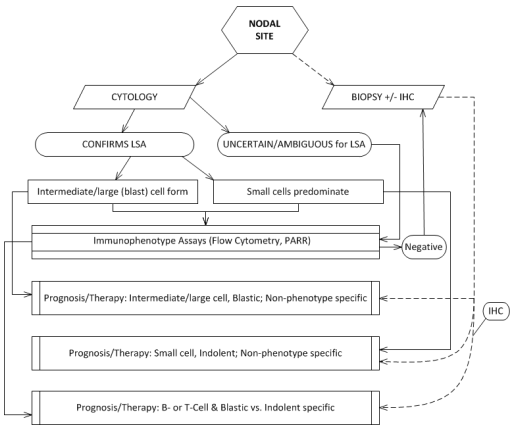
Courtesy of Dr. Anne Avery

NOTE: A polyclonal result (negative) does not rule out the presence of a neoplastic clone

Clonality (PARR) Assays: Specimen Requirements

- Aspirate tissue & transfer material into LTT
- Repeat until cloudy appearance to the EDTA
- Cytology slides (stained or unstained)
- Does not require viable cells – suitable for lysed material from glass slides or fluid.
- Some labs can perform from paraffin-embedded sections





Flow Cytometry and PARR: Summary

- Relative indications
- Advantages/disadvantages
- Prognostic value
- Comparative accuracy
  - AVIM Study Data
- Targeted therapy
- Advice from attending clinicians

Cytology Versus Biopsy: Comparisons of Accuracy

Andrew S. Loar, DVM, DACVIM (IM and O)

The evaluation of clinically abnormal tissues submitted for cytological analysis has for decades remained a simple and low cost diagnostic technique. In contrast to the acquisition of biopsy samples, material obtained for cytology examination generally requires less morbidity, time and planning. Only a small number of studies have demonstrated the relative value of cytologic, compared to histologic (biopsy), diagnoses in the same patient. The discussion below reviews research performed by clinical and anatomic pathologists to assist clinicians who must determine the accuracy of results derived from cytologic specimens.

Qualitative Determinations of Diagnostic Testing Accuracy

The diagnostic value of laboratory assays can be established using a variety of methods. For any discourse concerning the validation of a test, obligatory components include terms such as index test, reference standard, sensitivity, specificity, positive predictive value, negative predictive value, diagnostic accuracy, bias and disease prevalence. While the calculations and the statistical relevance for many of these parameters can seem complex, if not obtuse, precise definitions and consistent usage of the terms are crucial for evaluating studies described here.

For the purpose of this review the *index test* is the cytologic analysis, performed to decide if a patient has the disease of interest, and it is compared to the *reference standard*, also termed gold standard, which represents the histologic analysis of a biopsy specimen. *Diagnostic sensitivity* is the frequency a test is positive in patients that have the disease of interest; a test that has a high sensitivity has few false negative results and is suitable in screening for the presence of the disease. *Diagnostic specificity* is the frequency a test is negative in individuals that do not have the disease of interest; a test that has a high specificity shows few false positives and, thus, is suitable for confirming the presence of the disease. *Diagnostic accuracy* is the frequency a test correctly identifies a patient as having, or not having, the disease of interest; a test with high diagnostic accuracy reveals relatively few false positive or false negative results. Since an index test is often performed to identify more than one disease process, it is useful to defined the assay's diagnostic accuracy by determining how often the index test agrees, or correlates, with the reference standard. Thus, many studies discuss the frequency of concordancy (correlation) or discordancy. *Positive predictive value* (PV+) is the probability that a positive test result indicates the patient has the disease. *Negative predictive value* (PV-) is the probability that a negative result indicates the patient does not have the disease.

Of the above terms, only the positive and negative predictive values will vary dependent on the prevalence of the disease in question. Based on the respective formulae for calculating positive and negative predictive values, principally reflecting variation in the absolute numbers of all negative and all positive tests, if a disease is highly prevalent within the study population then the positive predictive value of the test increases, but its negative predictive value decreases. Conversely, if a disease prevalence is low then the positive predictive value for that test decreases and the test's negative predictive value increases. The concept of disease prevalence within a study population illustrates a potential for bias in virtually every clinical situation, and is highly relevant in the validation of cytologic testing. For example, neoplasia of canine long bones may be uncommon in the general pet population and, thus, a test (aspirate cytology) with good diagnostic specificity would still be associated with a relatively discouraging positive predictive value. However, in the population of dogs with radiographic evidence of a lytic or proliferative bony lesion the prevalence of a malignant mesenchymal tumor is very high, which, in turn, demonstrates a considerably increased positive predictive value for its cytologic evaluation. Similarly, when cytology is used as a screening assay in patients with a low chance of having an uncommon disease, the reliability (negative predictive value) of a negative cytologic study is considered to be very high.

As described above, a peculiar component of studies comparing cytology with histopathology is the concept of *agreement*. In contrast to use of the terms *specificity* and *sensitivity*, which reflect the accuracy of a test (cytology) seeking a certain condition, such as neoplasia or inflammation, calculation of a test's agreement is simply the proportion of cases where the diagnosis matches that of the reference standard. Many investigations distinguish between complete and partial agreement of a diagnosis where, for example, partial agreement indicates cytologic findings of malignant neoplasia, but no cell lineage is provided. Other examples of partial agreement include a cytologic diagnosis of neutrophilic inflammation versus a biopsy confirmation of acute (suppurative) cellulitis with fibrosis, or a cytologic diagnosis of undifferentiated carcinoma versus histologic evidence of adenocarcinoma, or a cytologic diagnosis of round cell sarcoma versus a biopsy confirmation of osteosarcoma. On many cases this degree of variation between cytologic and histologic interpretations may be clinical significant, however, during statistical analyses, studies often combine the complete and partial agreements into a single group, thus creating a more favorable test accuracy.

There are several inaccuracies inherent in the performance and evaluation of cytology and histopathology assays. The quality of both tests, and that of these comparative studies, are obviously dependent on the experience of the clinical and anatomic pathologists reading the slides. There can be significant variations in diagnoses between different pathologists evaluating the same tissues. Indeed, similar, if not less dramatic, differences have been identified when the same individual examines the same slides at different times. Several histopathology studies have reviewed interobserver variation, chiefly representing differences between first and second opinions, in the diagnosis of an amalgam of biopsy submissions. Significant diagnostic disagreements occurred in 10-20% of the cases, with clinically relevant partial (dis)agreement noted in nearly 50%. Also, variation in the type of specimen obtained for biopsy has been associated with discordant results; needle or core histology samples generally have revealed less accuracy than material obtained via excisional or wedge resection. These and other discrepancies call into question the term 'gold standard' with reference to histopathologic analysis and the difficulty in confirmation of true positive and true negative findings.

Nonetheless, the methodology of cytologic testing should likewise not be considered consistent. Every cytology specimen must be interpreted with the presumption that material submitted may not be representative of the aspirated lesion. This is self-evident when the sample is acellular or consists of only peripheral blood elements; however, in more cellular specimens a clinical pathologist may identify distinct cytologic findings, which are negative for specific disease, yet fails to suggest that the results may be inconclusive. This is highly relevant when comparing the value of cytology to biopsy because, in most investigations, the exclusion of inconclusive cytologic diagnoses markedly improves the determination of diagnostic accuracy. A cytology finding that does not confirm tumor, or any other definitive diagnosis, should not necessarily be considered a negative result if it is performed on, or identified as, a non-representative specimen.

Other limitations in cytologic evaluation include the distribution and accessibility of the lesion in question. Aspirates of focal, particularly external, masses generally yield more representative findings compared to material obtained from more diffuse lesions or those sought via intracavitary needle assaults. Similarly, ultrasound-guided aspirates are proven to be more accurate than when the needle is aimed with less direction. Finally, the size of the needle used, the aspiration technique and the number of slides prepared are all associated with variation in diagnostic accuracy and the reader is encouraged to review standard cytology texts to develop suitable methods of sample procurement.

In the past decade, researchers have adopted a distinctive set of standards for reporting of diagnostic accuracy - proposed under the acronym STARD – and subsequent publications of test comparisons are expected to adhere to these guidelines. Need for quality in the performance and reporting of studies that examine test accuracy cannot be overemphasized. By endorsing or discrediting a specific assay, a widely reported but poorly designed investigation can disrupt laboratory interpretations by a generation of clinicians. References are provided showing diagrams for the design of diagnostic accuracy protocols; these are helpful for readers to critique methods used for validating any new or alternative test. Generally,

the data in this chapter have been compiled from studies that followed the STARD group recommendations.

Comparison Studies: Cytology Versus Biopsy

Investigators have thus examined the diagnostic accuracy of cytologic analyses, compared to the presumptive gold standard of histopathology, for the evaluation of tissues in a number of selected anatomical locations. Various studies have addressed lesions identified from the skeleton, gastrointestinal and respiratory tracts, subcutaneous and dermal sites, liver, spleen, lymph nodes and prostate gland. For each site, the diagnostic sensitivity, specificity and accuracy of cytology will be described herein, as well as other significant factors relevant to the correlation of microscopic diagnosis and disease within each of these systems.

Bony Lesions

Compared to diagnoses derived from histologic analysis, using incisional (core) and/or excisional (generally via amputation) biopsy, cytology findings from radiographically identified bone lesions are variably accurate, particularly dependent on whether the process is neoplastic versus inflammatory. In several studies of more than 50 canine bony lesions, the vast majority of which were in long bones, cytologic evaluations were in agreement with biopsy results in approximately 70% of the cases. It is noteworthy that incisional biopsy results were in agreement with excisional biopsy findings in only 54% of the cases; examination of excisional bone specimens is considered the reference/gold standard. However, there was marked variation in the accuracy of cytology for identification of neoplastic compared with non-neoplastic conditions; more than 90% of the cytologic diagnoses of bony tumors were confirmed via biopsy, while less than 30% of cases with cytologic findings of non-neoplastic (generally inflammatory) disease were verified on histopath – the majority of these discordant cases were confirmed, via biopsy, to be neoplastic.

In summary, using needle aspirates of lytic/proliferative skeletal lesion, less than 10% of all cytologic diagnoses of neoplasia are shown to be non-neoplastic (low false positive rate), while more than 70% of all cases without cytologic evidence of neoplasia are ultimately confirmed to be tumor (high false negative rate). The factor that most significantly influences diagnostic accuracy is the overall cellularity of the cytologic specimen; samples with low to poor cellularity, which arguable could be interpreted as non-diagnostic, were most frequently associated with a poor correlation to subsequent biopsy analysis. In practical terms, the clinician can generally expect that a cytologic diagnosis of bony neoplasia from a highly cellular aspirate is accurate and reliable; in contrast, the majority of cytologic diagnoses of non-neoplastic (inflammatory) bony disease, particularly from samples of low cellularity, should be re-aspirated or biopsied to verify tumor.

Gastrointestinal Tumors

Compared to diagnoses using histology, generally from full thickness or endoscopically derived biopsies, cytology findings from gastrointestinal tumors show impressive accuracy. In several studies of more than 100 canine and feline gastrointestinal tumors, cytologic evaluations of samples with acceptable cellularity were in agreement with biopsy results in nearly 90% of the cases. The highest levels of agreement, with specificities and sensitivities of virtually 100%, were from cytology specimens representing impression smears of the biopsy samples, many of which were submitted as intra-operative slides.

Tumor types in these studies included gastrointestinal lymphoma, carcinoma and mesenchymal neoplasia (leiomyoma and leimyosarcoma). As stated above, specificity of cytology evaluation from fine needle aspirates of all tumors was nearly 100%. However the rate of false negative cytology results (sensitivity) varied by cell type; the highest sensitivity was found in the cytologic diagnosis of lymphoma (71%), followed by gastrointestinal carcinoma (63%) and mesenchymal tumors (44%). There has been no accuracy study published for non-neoplastic gastrointestinal lesions. It is noteworthy that investigations of

gastrointestinal biopsy results have showed significant variation in diagnoses when more than one anatomic pathologist reviews the specimens; one study found interobserver disagreement associated with more than 50% of intestinal lesions examined. Moreover, regarding the proposed gold standard for verification of gastrointestinal disease, many dispute the comparative accuracy of biopsy results derived from full thickness surgical excision versus those obtained endoscopically.

Respiratory Tract Lesions

The diagnostic accuracy of cytologic submissions from the nasal cavity and pulmonary tissue is generally good to excellent. In the most recent study of 28 dogs and cats with diffuse or focal lung lesions, more than 80% of the diagnoses obtained by aspirate cytopathology agreed with those found on subsequent histologic sections. Cytology specificity and sensitivity were both between 80-90%. This and other investigations found a similarly favorable accuracy examining neoplastic and non-neoplastic conditions. The least discordancy was seen when needle aspirates were performed with ultrasound guidance on focal or densely disseminated pulmonary lesions. These data contrast sharply with the extremely low sensitivity (high false negative rate) for cytologic examination of tracheal-bronchial lavage fluids in animals with pulmonary neoplasia.

Nasal cytology specimens derived from endoscopically-guided nylon brushes require less invasiveness and/or provide more representative results than those obtained from nasal discharge swabs, sinus flushing, blind needle aspirates or impression smears from surgical biopsies. In a study of 138 dogs with chronic intranasal disease, nearly 50% with neoplasia, brush cytology results showed a sensitivity of over 70% and a specificity of 99%. Many of the false negative cytologic diagnoses were attributed to the presence of increased numbers of mucosal epithelial inflammatory cells, which may have blunted the exfoliation of neoplastic elements. Although relatively few cases of lymphoproliferative disease were noted in this study, all forms of neoplasia, including those of epithelial or mesenchymal origin, were identified equally as well utilizing this technique. Moreover, the procedure correctly distinguished between neoplastic and non-neoplastic conditions in more than 85% of the patients. Brush cytology methods have shown a very similar accuracy (86%) in cats with intranasal disease. These studies suggest that adequate visualization of affected portions of the nasal sinus improved the yield and accuracy of the brush cytology technique.

Cutaneous and Subcutaneous Lesions

In the commercial and academic clinical laboratory, the most common sources of cytology specimens are cutaneous and subcutaneous sites, and include those of mammary origin. Studies with many hundreds of animals have generally indicated excellent correlation between cytology and biopsy findings, again emphasizing the advantage of lesion visualization. The most comprehensive investigation reviewed findings from nearly 300 canine and feline mass lesions, of which 175 were neoplastic. In this study the cytologic diagnosis was in agreement with histopathology results in 90% of the cases. In patients with neoplasia, cytologic analysis showed a sensitivity of 89%, a specificity of 98%, a positive predictive value of 99% and a negative predictive value of 68%.

Biopsy-confirmed neoplastic conditions that were not correctly identified by cytology consisted chiefly of well differentiated, albeit malignant, mesenchymal tumors co-existing with moderate to severe inflammation; these lesions were typically given a cytologic diagnosis of granulation tissue with pyogranulomatous inflammation. Indeed this discrepancy is quite common due to i) the inherent resistance of connective tissue tumors to exfoliate tumor cells, and ii) the development of variable to profound mesenchymal (fibrous) hyperplasia and dysplasia/atypia associated with focal inflammation. Similarly, these factors often result in an erroneous cytologic diagnosis of low grade sarcoma with inflammation when a subsequent biopsy indicates only granulation tissue.

In the study reference herein, a small number of benign keratin-containing or adnexal tumors were also diagnosed, cytologically, as non-neoplastic. However, the clinical behavioral distinctions between benign biopsy-confirmed cutaneous tumors such as pilomatricoma, trichoepithelioma, sebaceous adenoma and

basal cell tumor (now termed cutaneous trichoblastoma) are equivocal and arguably not different than that implied by a cytologic diagnosis of benign keratin cyst or adnexal structure proliferation. Interestingly, the cytologic diagnosis of certain non-specific round cell tumors showed the most variable and least favorable accuracy. While most forms of cutaneous lymphoma and histiocytoma, and all cases of mast cell tumor, were correctly diagnosed, a number of other lesions – specified only as round cell neoplasms - could not be more precisely identified without biopsy confirmation.

The management of mammary gland tumors in dogs and cats is frequently aided by aspiration cytology. In a study of 50 benign and malignant canine mammary tumors, cytologic diagnoses correlated with histopathologic findings in more than 85% of the cases. In animals identified with histologically malignant mammary lesions, pre-surgical cytologic examination revealed a sensitivity and specificity of 88% and 96%, respectively. Nonetheless, there are significant prognostic limitations comparing cytology versus biopsy findings. For example, a canine mammary tumor with a biopsy diagnosis of adenocarcinoma and no evidence of local or regional invasiveness is generally cured if completely excised. Likewise, tumors identified histologically as carcinoma in-situ rarely recur post-operatively and require no adjunct therapy. A far less favorable prognosis is offered for mammary carcinoma where blood or lymphatic vessel invasion is identified. Unfortunately, cytologic examination of aspirates from each of these three tumors may accurately render a diagnosis of malignant epithelial neoplasia (carcinoma), but cannot verify the presence of tumor invasion. Therefore, prediction of tumor behavior is ultimately dependent on evaluation of the excisional biopsy specimen. The reader is encouraged to review the literature for current and ongoing discussions regarding significant interobserver variation in diagnostic nomenclature and the often uncertain, if not confusing, correlation between a mammary tumor's histopathologic classification and its post-surgical behavior.

Feline mammary tumors reveal a considerably higher incidence of malignancy and, compared to canine mammary carcinomas, the vast majority show aggressive post-operative behavior. Thus, the cytologic diagnosis of feline mammary adenocarcinoma is considered highly accurate and generally predicts an ominous prognosis.

Liver Lesions

The ubiquity of utilizing abdominal ultrasonography in recent decades has dramatically increased the submission of guided visceral needle aspirates and core samples. Numerous large studies have examined the correlation between cytology and biopsy diagnoses from material derived from hepatic tissue. Here too, the gold standard is not well defined. Investigators have shown significant variation between histopathologic morphologic diagnoses when comparing needle (core) and wedge biopsy techniques, with a discordancy rate of higher than 50%. Moreover, in the same study, using the liver wedge biopsy method as a reference standard, interobserver differences were also common; three pathologists reading identical slides were in diagnostic agreement on only 65% of the cases. In most animals undergoing ultrasound-guided tissue procurement, the number of cytologic preparations is much higher than that of needle biopsy specimens, and the proportion of cases having wedge biopsies are considerably lower. This suggests liver biopsies are generally performed when the initial cytologic diagnoses are equivocal or non-contributory.

The most recent study examined cytology and biopsy findings from more than 700 dogs diagnosed via ultrasonography with focal hepatic lesions. More than 60% of the cases had only cytology performed, while 15% had only biopsy performed; the remaining, approximately 23%, had biopsy following cytology and these cases were the basis of the comparative study. While the presence of one or more focal lesions implies a similar distribution of disease, a significant problem interpreting cytologic accuracy in this study was the unlikelihood that a biopsy specimen from each case were derived from the same intrahepatic site as the cytology aspirate. Compared to biopsy diagnosis, cytology was most sensitive for the detection of hepatocyte vacuolar changes (58%) and neoplasia (52%) and lowest for identification of inflammation (31%) and hyperplasia (25%). Among patients identified with hepatic neoplasia, cytology was most sensitive for round cell tumor (60%) and nonhepatocellular carcinoma (54%), followed by

hepatocellular carcinoma (39%). Cytology positive predictive values for all tumor types were considered excellent at 87% (range 75-100%). This and several other studies strongly suggests that a cytologic diagnosis of liver neoplasia is accurate, although the absence of tumor cells does not exclude the diagnosis. Likewise, several researchers have shown that hepatic aspirates in cats with moderate to severe lipidosis can reliably identify the condition.

The specificity and, particularly, positive predictive value of hepatic cytology are less certain with diagnoses of inflammation, canine hepatic vacuolar changes and hyperplasia. Regarding the latter two morphologic findings, different studies reveal data suggesting mixed correlation with biopsy analysis, in large part because individual hepatocyte appearance may be more prominent, or considered more significant, in cytologic preparations than in those from histopath specimens. Most researchers conclude that a cytologic diagnosis of hepatic inflammation has an unfavorably low positive predictive value. This implies that cytology consistently over emphasizes the presence of significant suppurative or non-suppurative infiltrates. Admittedly, the degree of neutrophilia can be difficult to quantify cytologically, due chiefly to the inclusion of abundant peripheral blood elements and the uncertain proportion of blood-origin leukocytes. Obviously cytology cannot verify the location of the neutrophilic or mononuclear cell inflammation within the portal structural elements and, thus, histologic examination of tissue architecture is required to confirm the distribution of hepatic inflammation. However, until more definitive, prospective investigations are initiated, principally utilizing wedge biopsy techniques performed shortly after representative needle aspirate cytology from analogous anatomic sites, no further discussion can be offered.

Splenic Lesions

The indications for splenic aspirate include focal or diffuse parenchymal textural abnormalities, generally identified via ultrasonography, and staging for mast cell neoplasia. In spite of the vast number of ultrasound-guided splenic aspirates submitted to cytologists annually, only a single study comparing cytology results to those from histopathology has been published. An obvious drawback to this evaluation is that core biopsy procedures are less commonly performed on the spleen, in contrast to those from other abdominal viscera; thus, the gold standard of a confirmatory splenic biopsy is limited to animals undergoing splenectomy and/or abdominal surgery, or necropsy. Another variable was the significant, or uncertain, delay between the cytology aspirate and subsequent splenic biopsy.

Similar in virtually all comparison studies, most cytologic aspirates of the spleen are not followed by biopsy. Less than 15% of the patients with ultrasound-directed aspirates ultimately had splenic histopathology performed for verification. Presumably, cytology findings were considered sufficiently reasonable, when combined with the clinical features of the lesion(s) and other factors, to exclude the need for subsequent splenic biopsy. For example, the study selected very few patients with hyperechoic splenic nodules, a common ultrasonographic finding suggesting benign nodular hyperplasia; thus, cytologic examination of this lesion, yielding a diagnosis of splenic lymphoid hyperplasia, would be accepted by most clinicians and no further tissue sampling would be indicated. Yet, more ominous ultrasonographic lesions, such as a large focal mass or multifocal, hypoechoic nodules or evidence of diffuse to heterogeneous infiltrative disease, are less compatible with a cytologic interpretation of benign lymphoid hyperplasia, making an invasive biopsy well justified.

Of 32 cases of splenic disease, cytologic diagnosis in 85% showed complete or partial agreement with the biopsy diagnosis. Splenic aspirates from more than 90% of patients with non-neoplastic disease and approximately 75% of those with malignant tumors completely or partially agreed with the findings from biopsy specimens. Interestingly, multiple similar-appearing nodules were significantly more often associated with malignancy, while single lesions were more often benign.

In separate studies of splenic aspirates performed as part of tumor staging protocols for canine mast cell neoplasia, the utility of cytologic testing is less quantifiable. The specificity and positive predictive value of positive aspirates is certainly quite high, and the presence of several variable-sized aggregates of mast

cells throughout the specimen verifies disseminated mast cell tumor and a profoundly unfavorable prognosis. Likewise, the test sensitivity and negative predictive value of a negative result are considered to be very good. In North America, the prevalence of significant splenic mast cell infiltrates is low to extremely low (< 2%) in patients with solitary grade II de novo cutaneous mast cell tumors, including animals with abnormal or suspicious ultrasonographic findings; thus, irrespective of test accuracy, the use of splenic aspirate cytology on all animals presented for the management of mast cell neoplasia cannot be recommended.

Lymph Node Lesions

The indications for cytologic examination of lymph node aspirates include screening for tumor metastasis in sites proximal to neoplasia and determining the cause of node enlargement. No recent studies have been published reviewing the correlation of cytology with biopsy in the diagnosis of lymphoproliferative disease, in part because clinicians and pathologist so readily accept findings from needle aspirates of lymph node sites, particularly in patients with clinical manifestations characteristic of multicentric lymphoma. Verification of lymph node metastasis in animals with non-lymphoid tumors has been addressed in a study of 25 dogs with a variety of neoplasia. Compared to the gold standard of histological analysis, utilizing at least two longitudinal sections of the excised node, the accuracy of cytologic diagnoses was found to be excellent, revealing sensitivity and specificity approaching 100%. Concordance rates were equally good in patients evaluated for metastatic carcinoma, sarcoma or mast cell neoplasia. Indeed, cytologic findings indicated more favorable concordance rates than those of core biopsy specimens, and even successfully challenged the results of 'confirmatory' histopathology when only single longitudinal node sections where available.

A review of macroscopic metastatic patterns and lymph node architecture reminds the clinician that tumor cell distribution during early stages of dissemination principally involves the outer (cortical) regions of the lymph node, with eventual proliferation developing in the more central (medullary) portions. Thus, although not intuitive for most operators, sampling from sites parallel to the axis, and off-center, of the node maximizes the concentration of tumor cell infiltrate. The frequent demonstration of metastatic cells in enlarged, as well as palpably normal, lymph nodes emphasizes cytologic evaluation as a sensitive tool for tumor management.

An additional topic relevant to cytology submission is the often equivocal findings yielded when examining material from mandibular lymph nodes. Certainly in patients with neoplasia of the oral cavity or head and neck region the mandibular lymph nodes can be aspirated for the detection of metastasis. However, more than other peripheral node sites, mandibular lymphoid structures frequently sustain considerable antigenic stimulation, typically resulting from concurrent oral and nasal inflammatory disease. Increased relative numbers of reactive-to-immature lymphoid elements, neutrophils, mast cells and melanin-containing cells all may confound and confuse cytologic evaluation, particularly when the suspicious, expanded cell population matches that of the tumor-type being screened. Therefore, when patients are presented with peripheral multifocal lymphadenopathy clinicians are urged to provide samples from fellow lymph nodes, not limited to material aspirated from the submandibular sites.

In mast cell neoplasia, aspiration cytology is highly useful for tumor staging, particularly when performed on lymph nodes adjacent to tumor sites. However, the presence of mast cells in otherwise normal or hyperplastic nodes in non-tumor bearing patients threatens acceptable test specificity. Standardization of mast cell quantification has been necessary to distinguish the threshold of prognostically significant mast cell infiltration. Cytologic evidence of tumor metastasis has been defined as: i) the detection of moderate numbers of atypically granulated mast cells, ii) the presence of moderate numbers of mast cell clusters, with a cluster/aggregate determined to be greater than 3 cells, or iii) many mast cells without the evidence of concurrent lymphoid hyperplasia or inflammatory infiltrate. While any of these three criteria are considered definitive for mast cell tumor metastasis, the presence of lower numbers of mast cell aggregates downgrades the degree of suspicion to the more equivocal notation of 'possible' metastasis.

Prostatic Lesions

Prostatomegaly, masses and other conditions associated with the prostate gland are amenable to aspiration cytology. Results are generally comparable, if not superior, to those obtained from prostatic wash procedures or traumatic catheterization. Prostatic aspirates are ideally performed aided by ultrasound needle guidance and are associated with considerably less cost and complications than expected with traditional surgical biopsy techniques. Compared to the reference (gold) standard of incisional biopsy analysis, results from cytology specimens are quite accurate.

In a study of 23 dogs with prostatic disease, cytologic diagnoses agreed with histologic evaluation in more than 90% of the cases examined, with partial agreement in the remaining cases. In dogs with prostatic neoplasia, either adenocarcinoma or transitional cell carcinoma, sensitivity and specificity were at, or near, 90%. Indeed, in at least one case, cytology identified a malignant neoplasm that was not verified on initial biopsy, but was subsequently found at necropsy. In addition to malignant tumors, prostatic conditions found on cytology included benign prostatic hyperplasia, prostatic cyst formation, prostatic squamous metaplasia and prostatitis. Interestingly, this study showed an improved cytologic ability to identify bacterial organisms in dogs with septic prostatitis, compared to biopsy. Similar to data elsewhere the number of cases studied, selected because each had cytology performed as well as a biopsy, was 3-fold fewer than the number of cases where only a cytology was submitted. This implies that aspirate cytology is likely a principle, if not definitive, method for disease diagnosis in the majority of animals with prostatomegaly.

Other Aspirate Sites

Tissues and anatomic locations not otherwise addressed in these clinical studies include extra-visceral regions of the thoracic and abdominal cavities (and effusions within these spaces), the central nervous system and much of the genitourinary tract. Predictably, aspirates of parenchymal tissues and other solid tissue organs are most accurate when needle entry is visualized or guided, and when the specimen is of adequate overall cellularity. Cytologic diagnosis of neoplasia in these sites generally correlates with biopsy findings in a significant majority of patients, particularly with regard to lymphoproliferative disease and malignant epithelial neoplasms. A negative diagnosis for tumor is considered reliable for most of these tissue sites, with the notable exception of body cavity effusions. Although neoplasia is a frequent cause of a pleural or peritoneal modified transudate, evaluation of fluid specimens in more than 50% of these patients reveals no evidence of tumor cell exfoliation. In animals with non-neoplastic disease at these sites, and in contrast to data from liver aspirate studies, cytologic demonstration of inflammatory infiltrates is generally considered to be specific; the absence of significant inflammation also usually correlates with biopsy results.

Summary

The numerous variables described from the comparison studies above suggest that clinicians should not necessarily rely on published determinations of the accuracy of cytologic aspirates when pondering the justification for needle aspiration versus biopsy. The reader is reminded that comparison studies may consistently show a bias toward poorer accuracy, because a large proportion of the data is collected from patients with equivocal cytologic findings or in those where the disease is likely to be severe. Perhaps the most valuable information described here provides a list of specific tissue conditions that can be more, or less, reliably identified using cytology techniques. Cytologic findings associated with marginal accuracy, and particularly those diagnoses carrying grave prognostic implications, should be confirmed with histopathology. In addition, when direct visualization of the aspirated mass is not possible, advanced imaging techniques that aid in the identification of otherwise occult lesions increase the diagnostic performance of cytology. Qualitative interpretation of the initial cytology findings is also crucial. Results may be based on low cellularity or errant aspirate, slide making and staining methodologies and, therefore, always must be considered as potentially non-representative of the lesion. Conversely, discrepancies inherent to biopsy evaluation, particularly in certain tissues, often invalidate histopathology

as an accepted gold standard. Finally, clinicians should query whether these and future comparative studies give sufficient credit to the performance of cytology on the many, many lesions where the cytologic diagnosis is the sole determinant of appropriate clinical management.

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Molecular Diagnostics

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The fledgling field of veterinary molecular diagnostics is based on the identification of selected tumor cell features, including surface proteins, nucleic acids and other highly specific markers, to improve diagnostic management, to clarify tumor behavior and to customize treatment choices. While the assays briefly described here, to include flow cytometry, clonality testing and gene expression (c-kit), have been available for only a few years, they have profoundly enhanced the practice of oncology; and they are underutilized by the veterinary community. The purpose of this discussion is to review these methodologies and the indications for their usage, to address pertinent sample submission techniques for each, and to offer limited interpretative advice. For the management of common hematopoietic and lymphoproliferative neoplasms, and combined with traditional microscopic tissue analysis, these newest diagnostic tools are predicted to become as essential as they are elegant.

Flow Cytometry – Immunophenotyping of Lymphoproliferative Disease

Methodology and Sample Procurement

Flow cytometry determines selected, individual cellular characteristics by directing a light (laser) beam toward a single-file line of cells within a fluid and measuring the diffraction of the laser associated with each cell. The addition of dye-labeled antibodies, designed to bind specific cell proteins, identifies clusters of differentiation (CD) molecules when the instrument's light ray reacts with the complex, creating a measurable wavelength of energy. This technique can thus determine cell size, nuclear/cytoplasmic features and specific (CD and other) surface markers, as well as the relative number, of virtually every variety of leukocyte (neoplastic or otherwise) within a fluid specimen (Table 1).

The limitations of flow cytometry require a sufficient quantity of intact and viable leukocytes with minimal cell clumping, to be suspended in fluid and submitted to a facility proficient in the performance and interpretation of the analyses. Clinicians originally were restricted to submitting biologic fluids, generally consisting of anticoagulated whole blood, liquid bone marrow specimens or body cavity effusions. However, evaluation of material in solid tissue, such as that from lymphoid structures and visceral sites, is also possible using needle aspirates placed into appropriate fluid media.

Compared to the traditional methods for immunophenotyping of lymphoproliferative disease – that is, immuno-histochemical (IHC) or immuno-cytochemical (ICC) staining of biopsy or cytology preparations – flow cytometry performed on fine needle aspirates of solid tumors is an appealing alternative. This technique is simpler and far less expensive, while providing a much faster turn-around time, than the procurement of a biopsy with standard histologic evaluation and subsequent IHC processing. Compared to ICC analysis of a previously submitted cytology specimen, flow cytometry may represent a similar cost and clinical effort, but generally provides a more comprehensive, quantitative evaluation and interpretation.

Clinicians attempting needle aspiration of lymphoid tumors for immunophenotyping are encouraged to use vigorous and repeated needle thrusts while maintaining negative pressure and aiming toward the lesion, when prudent, in several alternative tangential directions. Repeating an often unheeded plea from cytologists, when aspiration sites require ultrasonographic visualization, it is critical that cutaneous needle entry avoids ultrasound gel. Immediately upon needle withdrawal, the contents of the needle, as well as any material within the hub and barrel of the attached syringe, are expelled into a tube containing an appropriate sterile transport media. This media, generally a solution of 10% fetal bovine albumin, may be provided by the flow cytometry facility, although an acceptable facsimile can be achieved using sterile saline (0.9% NaCl) combined with the patient's serum in a 9:1 ratio.

Guidelines for storage and shipping of flow cytometry samples are crucial to acquiring valid data. The following instructions are designed to maximize the proportion of viable cells submitted for the assay: i) Specimens should be refrigerated as soon as they are taken from the patient; ii) The specimen tube(s) should be placed into an insulated container holding cold packs, being careful not to place tubes directly onto the frozen material; iii) Rubber stoppers must be secured for shipping, ideally by establishing a vacuum within the tube;
iv) Specimens should be shipped to the destination facility for next day delivery, assuring the package does not arrive over a weekend or holiday – this often creates a dilemma regarding submission of samples obtained Friday-Sunday; in these cases laboratories typically advise to hold such specimens at the originating clinic until next day delivery is possible.

To reiterate, insuring a thorough and valid flow cytometry study is based on offering the instrument as high a concentration of intact and viable nucleated cells as is practicable. Although evaluation of as few as 50,000 individual cells may be sufficient for effective analysis, a variety of factors suggests that an estimated cell population of 250,000 to 500,000, per specimen, is optimum. For leukemic blood samples, where nucleated cell counts often exceed 50,000 per ul, this translates to considerably less than $1/10^{th}$ of one ml (milliliter) of whole blood. Less cellular specimens, however, including pleural/peritoneal effusions, cystic transudates or cerebrospinal fluid, generally require submission of larger sample volumes, particularly when handling, storage and transportation of the specimen threaten the viability of a significant proportion of their cellular elements.

As of early 2014, flow cytometry specimens are analyzed at a number of university and corporate industry-based commercial laboratories in North America: see the list provided under the web-bibliography at the end of this chapter. Turn-around times and costs differ considerably between facilities, varying from 3 to 15 business days and $140-300 (US), respectively. Throughout this chapter, in part due to space limitations, the text intentionally oversimplifies the identification of T- vs. B-cells, and offers few detailed examples of the CD (or other) marker findings derived from flow cytometry analyses; more complete descriptions can be found within the suggested reading material. Nonetheless, laboratories competent in molecular diagnostics consistently provide comprehensive quantification and interpretation of data from each submission. Lastly, in the following discussion, analyses and interpretation of canine and feline samples are generally considered similarly. The facilities that offer these assays have a larger menu of, and more experience using, anti-canine antibodies, although flow cytometry and clonality testing are reliably performed on most cat samples. Typically, technical limitations regarding feline leukocyte immunophenotyping, if relevant, will either be addressed in the literature provided on each lab's web-site or in the reported patient results .

Indications - Flow Cytometry

The clinical justification for any diagnostic test or procedure is based on the expected value of the knowledge acquired. If, irrespective of the results of a particular assay, there is to be no difference in the discussion, understanding or management of the disease then, perhaps arguably, the test should not be performed. Alternatively, even in the face of ambiguous prognostic and therapeutic implications, compilation of data relevant to the study of these tumors resonates with many clinicians. Additional factors in the choice of diagnostic management include the inclination to simplify and economize sample submission.

Flow cytometry is principally indicated for the characterization of the following conditions: 1) Peripheral (circulating) well differentiated lymphocytosis (absolute lymphocyte counts persistently greater than 10,000-15,000 per ul); 2) The presence of unidentified circulating blast forms (absolute blast cell count greater than 2,000-3,000 per ul);
3) Cytologically or histologically confirmed diagnoses of small, intermediate or large cell forms of (solid tissue) lymphoproliferative disease; 4) Cytologically suspicious lesion(s) containing an expansion of either large, blastic or small, homogenous lymphoid elements;

5) Suspected immune-mediated hemolytic anemia and immune-mediated thrombocytopenia.

1) Peripheral (circulating) well differentiated lymphocytosis: Cytologically it may be difficult to distinguish between a well differentiated (small cell) lymphocytic leukemia, including chronic lymphocytic leukemia (CLL), and non-neoplastic forms of reactive lymphocytosis, chiefly when absolute lymphocyte counts are less than 15,000-20,000. Although the prognostic and therapeutic significance of T- versus B-cell forms of CLL may be arguable, the value in distinguishing well differentiated lymphocytic leukemia from non-neoplastic forms of lymphocytosis is clear. Immunophenotyping, performed on animals with CLL, generally reveals a homogenous population of either B- or T- cells, while animals with non-neoplastic causes of lymphocytosis should demonstrate a heterogeneous population of B- and T-cells, the latter including an admixture of T-helper and cytotoxic T- lymphocyte subsets. Other subtypes of chronic (well differentiated) lymphocytic leukemia include one that has been identified with an aberrant (non-CD4 and non-CD8) T-cell phenotype. Flow cytometry has also been recommended in animals suspected of being immune-deficient; indeed, for human beings, this assay is performed more frequently to determine CD4/CD8 status in HIV-positive individuals than for any other purpose.

2) The presence of circulating blast forms (absolute blast cell count greater than 2,000-3,000 per ul) - including leukemias where the abnormal leukocyte is of uncertain lineage: In a patient with cytologically confirmed circulating lymphoblastic elements, it is prognostically useful to distinguish a primary leukemic process from advanced stage (V) multicentric disease. The absence of significant disseminated soft tissue tumor in the presence of severe marrow infiltration and a profound peripheral lymphocytosis generally supports the diagnosis of acute lymphoblastic leukemia (ALL). Immunophenotyping of the circulating lymphoblasts should distinguish between T-cell versus B-cell forms of ALL, and, thus suggests variable options for treatment. Alternative, and more ominous, forms of acute (blastic) leukemia show a variety of non-lymphoid phenotypic characteristics. These most commonly originate from myeloid, monocytic, myelomonocytic or megakaryoblastic lineages. Immunophenotyping of acute leukemias, particularly when cytologic evaluation is unable to differentiate lymphoid from non-lymphoid tumor elements, is crucial to determine prognosis and therapy.

3) Cytologically or histologically confirmed diagnoses of small, intermediate or large (blast) cell forms of solid tissue lymphoproliferative disease: For more than a decade, canine lymphosarcoma has been considered a heterogeneous disease, with different clinical outcomes often correlating with different morphologic findings. Classically, the variable forms of lymphoma have been distinguished based on histologic evaluation of tissue architecture and, when available, immunophenotyping of formalin-fixed paraffin-embedded biopsy sections. However, many canine patients with lymphoma are not currently managed with the benefit of histopathology. Based on recent experiences of veterinary oncologists throughout the US and a poll of several commercial veterinary pathology facilities, considerably less than 20% of canine lymphoma cases will have both histopathology and cytopathology data available when the initial treatment plan is discussed. Indeed the vast majority of cases are managed based solely on a diagnosis derived from fine needle aspirate cytology. Moreover, immunophenotyping, using any methodology, currently is performed on less than one lymphoma patient in 10.

Notwithstanding costs, the principal argument used against the routine evaluation of lymphoma markers is that clinicians have not been sufficiently influenced by the literature, or their own clinical experience, to recognize advantages in the management of the disease based on verification of a T-cell, versus B-cell, tumor population. Generally, cytologic examination is able to distinguish between small (well differentiated) and intermediate or large blastic variants of lymphoma. While most small cell forms of the tumor respond favorably to therapy protocols that are quite distinct from those used against large, blast cell lymphoma, relatively few investigations have examined and confirmed differences in tumor control and survival correlating with the phenotype of lymphoblastic disease. Certain, albeit uncommon, features are shown to be strongly associated with unfavorable outcomes,

particularly sparse expression of a major histocompatibility complex (MHC II) on large blastic B-cells. These studies, and other pertinent reviews, are identified in the reference reading material herein. Gradually, additional information may ultimately be offered suggesting the value of immunophenotyping for all clinical presentations of canine lymphosarcoma.

4) Cytologically suspicious lesion(s) containing an expansion of large and/or blastic lymphoid elements: Fine needle aspirates of peripheral and visceral lymph nodes, and those from extra-nodal sites, frequently reveal increased numbers of enlarged, immature and/or monomorphic lymphoid elements, though not in sufficient proportions to confirm lymphoproliferative disease. In patients showing one or more enlarged peripheral lymph nodes, needle aspirates comprised of less than 50% obvious blast forms, particularly when combined with a mixed population of small or reactive lymphocytes, are generally interpreted as 'suspicious for, but not confirmatory of' lymphoid neoplasia. This result is likewise common with specimens from splenic, hepatic or gastrointestinal lesions where a relatively low proportion of enlarged and/or immature lymphocytes is considered noteworthy - yet the cytologist is unwilling to verify lymphoma. An analogous dilemma pertains to cytologic evaluation of mediastinal masses, particularly in cats, where it is often difficult to eliminate the possibility of small cell lymphoma in favor of thymoma. For each of the examples above, tumor confirmation has traditionally committed the clinician to the expense, time and risks of acquiring a biopsy specimen. Flow cytometry, performed on needle aspirates from any of these lesions, is proven to be an effective and less costly alternative to histopathology and IHC. With material submitted in an appropriate fluid media, analysis will typically distinguish between lymphoma and non-neoplastic lymphoid infiltrates and, moreover, generally demonstrates the size and phenotype (B versus T) of the principle cell of interest.

5) Suspected immune-mediated hemolytic anemia (IMHA) and immune-mediated thrombocytopenia (ITP): Given the traditionally marginal reliability of Coombs testing, a diagnosis of canine IMHA is most commonly suggested based on evidence of red cell agglutination and/or spherocytosis in patients with regenerative anemia. Recently, the development of a novel flow cytometry assay that demonstrates erythrocyte membrane binding of IgG, IgM and complement has shown impressive sensitivity and specificity. When this test is widely available it likely will become the benchmark for confirmation of immune associated hemolysis. There is also an analogous assay that identifies IgG binding on platelet membranes; the presence of increased surface IgG in thrombocytopenic patients strongly supports a diagnosis of either primary or secondary ITP. Most facilities performing flow cytometry assays offer this test. False positive results are reported due to in vitro (post-collection), non-specific IgG platelet binding, so specimens must be evaluated less than 48 hours from sampling.

Clonality Testing – Polymerase-Chain-Reaction (PCR) for Antigen Receptor Rearrangements: PARR – and Lymphoproliferative Disease

Methodology and Sample Procurement

The process of PCR-based testing enables specific portions of a patient's DNA to be identified and characterized, via logarithmic replication (amplification) and subsequent analysis, allowing the demonstration of selected genes and gene mutations from relatively small portions of cellular material. In veterinary oncology, the most frequently ordered, and widely available, PCR assays include those used: i) to determine clonality, specifically the PARR test; and, ii) to identify c-kit gene mutations.

Analysis for c-kit mutations, broadly useful in addressing prognosis and therapy of canine mast cell tumors, typically is performed on formalin-fixed and paraffin-embedded sections of biopsy specimens. Alternative sample submission options, such as that using material derived from needle aspirates, have been investigated but are not currently advocated by commercial laboratory facilities.

Clonality testing using the PARR assay seeks to determine if the majority of elements in a lymphoid population are derived from a single neoplastic clone. The procedure demonstrates genes specific to B- and T-cells, specifically immunoglobulin receptor genes on B-cells and T-cell receptor genes on T-lymphocytes. In a benign admixture of lymph node constituents PARR analysis would be expected to identify multiple sized PCR products representing a variety of T-cell receptor and immunoglobulin (B-cell) receptor genes. In contrast, the presence of a single sized PCR product reflective of either a T-cell receptor gene or an immunoglobulin receptor gene would verify a tumor of T- or B-cell origin, respectively.

The accuracy of PARR assays may vary between facilities, and it is incumbent upon each laboratory to indicate the sensitivity and specificity of their procedure. In most laboratories this assay's specificity is greater than 90%, implying that virtually all positive results verify a neoplastic cell clone. However, a modest percentage of these cases may represent a malignant myeloid tumor, rather than lymphoma. In addition, PARR testing may demonstrate uncommon phenotypic forms of lymphosarcoma that reveal aberrant rearrangements, that is, those containing a clonal presence of lymphocytes with both T- and B-cell receptor genes. The assay's sensitivity may be less impressive, documented to be between 65-80%, dependent on the facility. False negative results using PARR testing may be due to several technical factors, one of which is the assay's inability to detect low proportions of tumor cells against a larger background of normal lymphocytes.

PARR can be performed on virtually any specimens that contain cellular DNA. This includes whole blood, solid tissue aspirates, cavity effusions, bone marrow and cerebral spinal fluid (CSF). Samples may be submitted as a biologic fluid or on glass slides; slides previously stained for cytologic evaluation are also usable. Analogous to limitations addressed regarding flow cytometry submissions, adequate numbers of cells are necessary, although they do not need to be viable for analysis. Cellularity adequate for clonality assays is defined to be greater than 50,000 total cells; on a slide containing a consistent distribution of material over at least 50% of its surface, this equates to approximately 100 nucleated cells per low power (10x objective) field. Although many facilities can extract DNA from formalin-fixed, paraffin-embedded tissue on glass slides, the discussion here presumes that no biopsy has been obtained. Fluids (blood, bone marrow, effusions and highly cellular CSF) should be held at 4°C until shipment; slide samples containing material from tissue aspirates should be stored at room temperature. Expedited delivery may improve the timing, but not the reliability, of the test results.

Indications - PARR Testing

There are distinct similarities between the clinical indications to perform the PARR assay and those addressed earlier regarding flow cytometry. The principle value of the PARR assay is to distinguish a non-neoplastic population of lymphoid elements from lymphoma when histologic or cytologic evaluation fails to make an unequivocal diagnosis. Inconclusive microscopic interpretations are frequent in the pathologic evaluation of small cell lymphoma, also called indolent lymphoma, which comprises a variety of tumors from both B-cell and T-cell lineages. In these forms of well differentiated (small cell) lymphoma, identification of clonal expansion and the determination of immunophenotype verifies a diagnosis that was not otherwise available from initial cytology or biopsy analysis.

Another compelling, and common, example where the PARR assay is considered useful includes the cytologic examination of a single enlarged node, especially at the submandibular region, or one draining a site of chronic inflammation. In these cases, the presence of a low, yet suspicious, proportion of enlarged (intermediate-sized or larger) lymphocytes, or the expansion of a monomorphic, but small cell, lymphoid population typically yields a cytologic interpretation implying the findings are 'suggestive of, but not diagnostic for lymphoma'. Submission of the same slides for PARR assay is recommended in an effort to confirm lymphoproliferative disease and provide immunophenotype data.

Similarly, needle aspirates, from any site, revealing poorly differentiated round cells create a diagnostic problem for all cytologists. Although most forms of anaplastic round cell neoplasia can be distinguished morphologically, atypical lymphoma remains on each differential list. For these cases, the submission of molecular diagnostics, using either PARR testing or flow cytometry, is an efficient and reliable method to eliminate or confirm lymphosarcoma.

A second indication for use of the PARR assay is to demonstrate and characterize recurrent lesions in patients with previously diagnosed lymphoproliferative disease. Given a documented sensitivity of detecting tumor cells, against a background of non-neoplastic lymphocytes, in concentrations as low as 1:100, this test may be useful in identifying early nodal relapse or the development of circulating leukemia. Lastly, in occasional tumor-bearing patients, lymphoproliferative disease may transition from one immunophenotype to another. This may be confirmed by comparison of PARR assay results obtained at different time points.

The indications for flow cytometry and the PARR assay are perhaps best illustrated using an algorithm (Table 2: described in lecture). Notwithstanding the similar indications for the use of PARR assays versus flow cytometry, the advantages of PARR are: i) Specimens submitted for PARR testing may include the same slides originally provided to the cytologist, and these may have previously been stained using Wright's-Giemsa or Diff-Quik processing; ii) Unstained slides from needle aspirates, blood or fluid representing body cavity effusions often contain sufficient cellular DNA for testing, without the need to provide viable cells. Disadvantages of the PARR assay, compared to flow cytometry, include a decreased sensitivity, described above. Therefore, inability to confirm lymphoproliferative disease with the PARR assay should be pursued with the submission of additional material for flow cytometry.

Conclusions

Ultimately, the alchemy of tumor cell morphology, microscopic architecture, immunophenotype and clinical reckoning combine to generate the most comprehensive data base for each patient's neoplasm. The goal for clinicians managing possible lymphoproliferative disease is to choose a collection of sensitive and specific tests that confirms and best characterizes the tumor. Evaluation of tissues using standard cytologic or histologic processing, followed, where appropriate, by one or more of the molecular diagnostic techniques briefly described here, provides information with significant prognostic and therapeutic implications. With time, demonstration of the clinical relevance of these findings will mandate the appeal of each assay.

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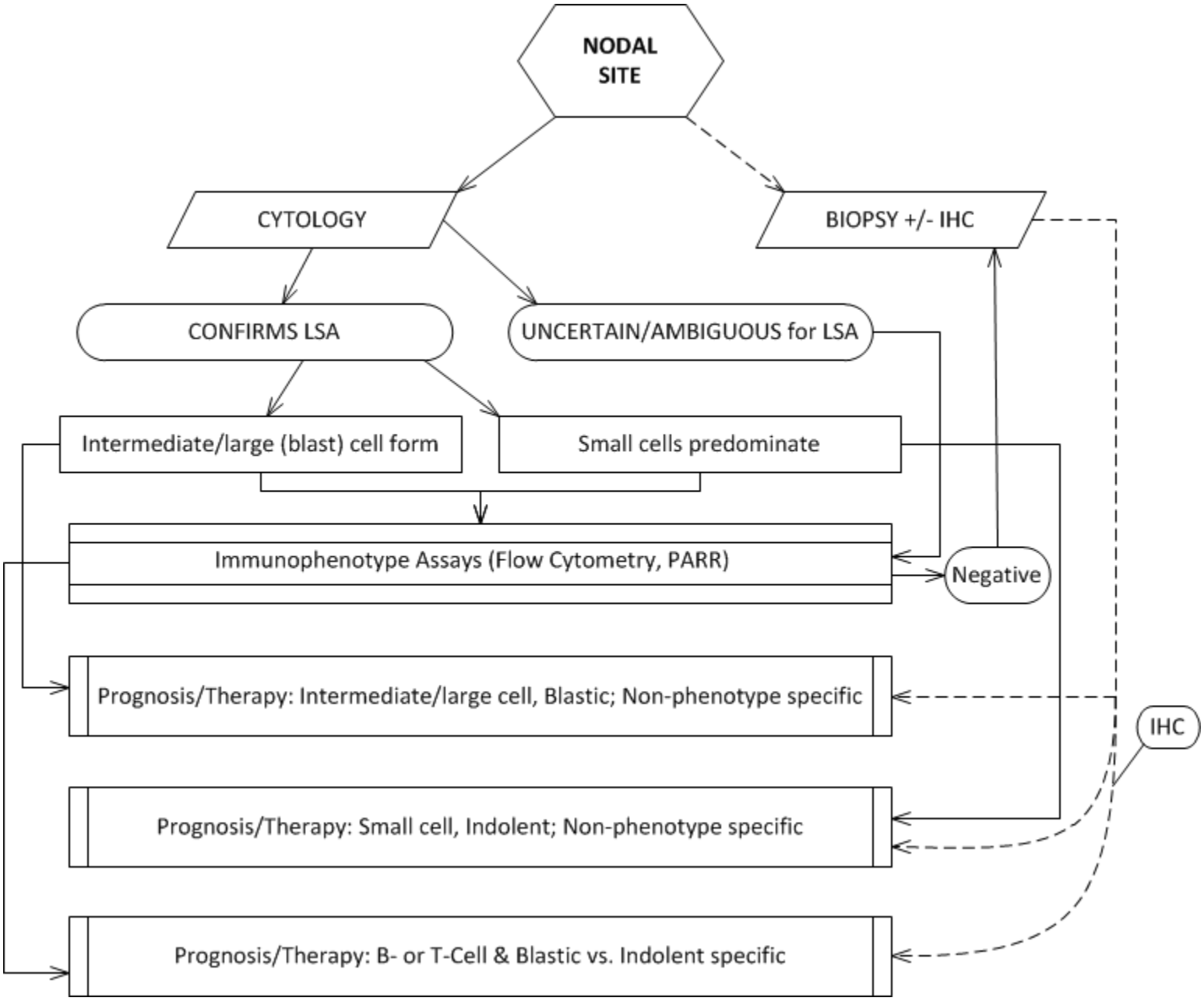
TABLE: 1

T-cell (all) – CD3
T-cell (all) – CD5
T-cell (Helper T) – CD4
T-cell (Cytotoxic T) – CD8

B-cell – CD21
B-cell – CD22
B-cell – CD79a
B-cell –MHC II

Monocytes – CD14
Leukocytes (all) – CD18
Leukocyte stem cells – CD34
Leukocytes (all) – CD45

Caption for Table 1: Cluster of differentiation (CD) molecules and other surface or cytoplasmic markers available for leukocyte identification. NOTE: Each commercial laboratory panel may provide a different complement of markers; these also vary dependent on the species – canine vs. feline**.**


NODAL SITE
CYTOLOGY
BIOPSY +/- IHC
CONFIRMS LSA
UNCERTAIN/AMBIGUOUS for LSA
Intermediate/large (blast) cell form
Small cells predominate
Immunophenotype Assays (Flow Cytometry, PARR)
Negative
Prognosis/Therapy: Intermediate/large cell, Blastic; Non-phenotype specific
IHC
Prognosis/Therapy: Small cell, Indolent; Non-phenotype specific
Prognosis/Therapy: B- or T-Cell & Blastic vs. Indolent specific