Canine Lymphoma Staging Tests

Are they really done anymore?

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Consultant for Heska
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Live from Boulder, Colorado

HESKA

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Structure of this talk:

- Introduction
- Review of Lymphoma
- Available Staging Tests
- Discussion of Practical Real -World Situations
- Conclusion

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A little about me:

- Born and raised in Bethesda, MD
- University of Pennsylvania
  - for Undergrad Engineering, 1995,
  - Veterinary School, V'02,
  - Oncology Residency '03-'06
- Private practice since
  - NYC
  - Miami when
  - 2019 - I moved to Boulder, CO!

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Treeline Veterinary Cancer Care

- Started in 2020
- Small boutique bond-centered medical oncology practice
- Just me and my team
- Outpatient
- Spa-like feel
- Slightly, but not completely, open concept

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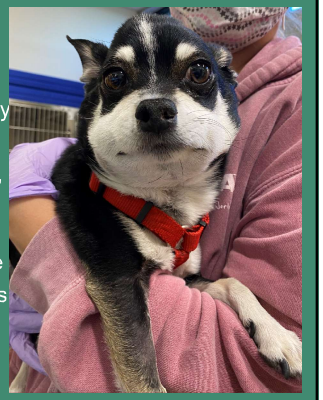
Canine Lymphoma

- Most common cancer we see in dogs
- AKA Lymphosarcoma
- Disease of Lymphocytes, a WBC involved in immunity
- Considered Systemic in Nature
- Usually treated medically (chemotherapy, or steroids, or both)
- While often referred to an Oncologist,
- Many dogs with lymphoma are still treated in practice
- Many Oncologists prefer that diagnosis and staging is done prior to referral



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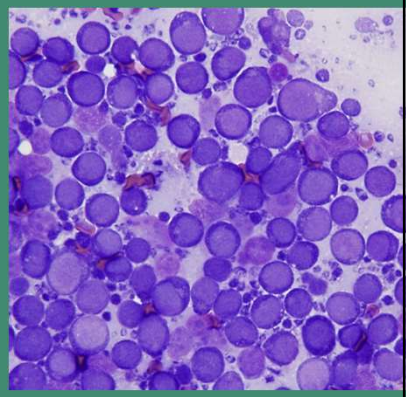
Diagnosis

- Fine needle aspirate/Cytology adequate in most cases
- Can even get with a touch prep (mucosal surfaces on external PE)
- Touch prep of a surgical sample
- I always look at mine in-house, and still….
- **I always** send out for confirmation



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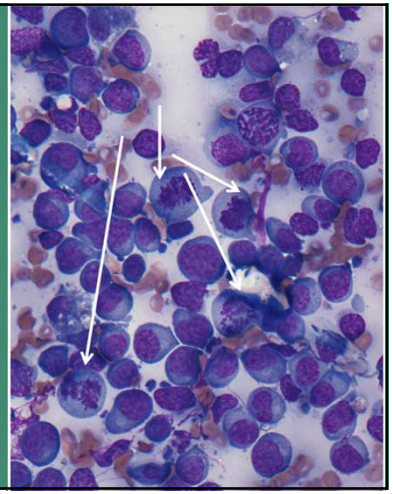
Diagnosis

- May not see criteria of malignancy, but often lots of mitotic figures
- Over 50% lymphoblasts
  - Lymphoblast > neutrophil
- May see a perinuclear clearing – especially in B cell Lymphoma, corresponds to hand mirror appearance



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Diagnosis

- When the aspirate doesn't provide the diagnosis
  - Biopsy
    - Small (lymphocytic) / intermediate sized cell
    - Tru-cut often adequate or can excise a node
  - Flow cytometry
    - separates cells based on their DNA, charge, or cell surface markers
  - PCR (AKA PARR)
    - Will test for clonality

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Staging Lymphoma

- After obtaining a definitive diagnosis, staging is the next step in developing a treatment plan.
- Staging determines both the ***distribution*** and the ***extent*** of the disease

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Rational for Staging

- Allows us to determine a patient's health status
- Allows us to safely use chemotherapy drugs
  - Vincristine, Doxorubicin – metabolized through liver
  - Cyclophosphamide – activated in liver
  - Cyclophosphamide, often given with furosemide, which is given with care in renal failure.
- Allows placement into WHO category of staging

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WHO (World Health Organization) Staging System for Canine LSA

I - A single lymph node involved

II - Regional Lymph nodes involved on one side of the diaphragm

III - Generalized lymph node involvement on both sides of the diaphragm

IV - Liver and/or splenic involvement

V - Bone marrow infiltration, or involvement of extranodal organs (e.g. skin, CNS, Lungs)

Each numbered stage is further divided into 2 substages:
- Substage A – patient feels well
- Substage B – patient is ill

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Which Staging Tests are Historically Performed

- Routine Lab work (CBC, Chemistry Profile, Urinalysis)
- Multiple Lymph Node Aspirates
- Chest Radiographs
- Abdominal Ultrasound, with organ aspirates
- Bone Marrow Aspirate
- Immunophenotyping

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Complete Blood Count

- Anemias
  - Anemia of chronic disease
  - Regenerative anemia (GI LSA) may indicate blood loss
  - Hemolytic Anemia (IMHA secondary to LSA)

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Complete Blood Count

Changes in WBCs
- Neutrophila
  secondary to inflammation
- Neutropenia
  secondary to bone marrow involvement
- Monocytosis
  could be an indication of circulating blasts especially if read on in-house analyzer
- Lymphocytosis
  could have concurrent CLL (Chronic Lymphocytic Leukemia)
  circulating blasts

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Complete Blood Count

Changes in Platelets

Many older dogs have thrombocytosis – indicating of inflammation or Cushing's

Could have a thrombocytopenia
active bleeding (i.e. – GI involvement)

Concurrent ITP, etc.

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Chemistry Panel

Invaluable for staging

- **Often normal**
- **But key indicators are:**
- **Renal values**
- **Liver Values, especially bilirubin**
  - **Could necessitate chemotherapy dose changes or change of the order of chemotherapy drugs**
- **Calcium – could indicate T-cell LSA**

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Urinalysis

- **Check for concurrent UTI**
- **Check kidney function**

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Thoracic Radiographs

When taking radiographs as part of staging, often 2 views are adequate.

4 typical patterns:

- Pleural Effusion
- Cranial Mediastinal Mass
- Lymphadenopathy
- Diffuse Pulmonary Infiltrates

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Thoracic Radiographs

- Beyond localizing the Lymphoma there are other reasons for doing thoracic radiographs
- Evaluation of heart size
  - undergoing treatment with Doxorubicin
  - Doxorubicin can cause cardiomyopathy
- Evaluation of pulmonary health
  - Tanovea is a newer medication for canine Lymphoma
  - Has been associated with causing pulmonary fibrosis
  - Not recommended in Westies
- CXR are good baseline in both cases if family are going to pursue treatment containing doxorubicin or Tanovea

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Thoracic Radiographs

INSERT POLL QUESTION HERE:
What is the most common way LSA presents in the lungs?

a) pleural effusion,
b) lymphadenomegaly,
c) diffuse pulmonary involvement,
d) cranial mediastinal mass,
e) normal thorax

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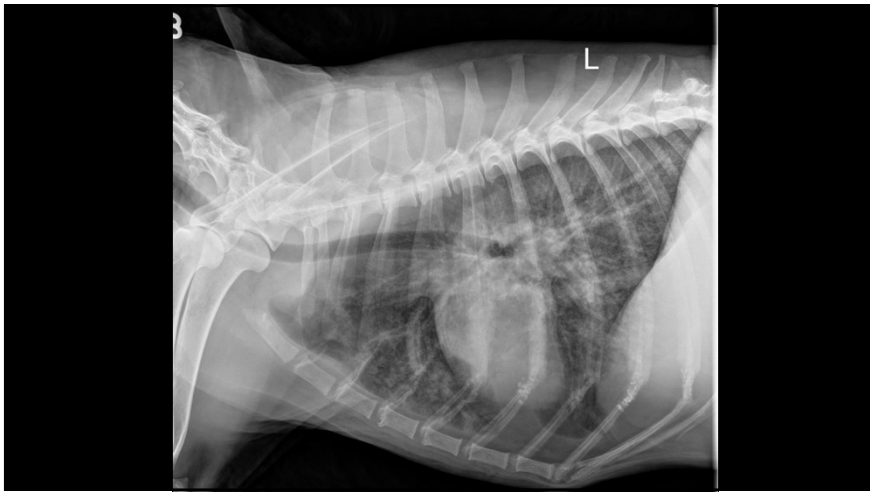

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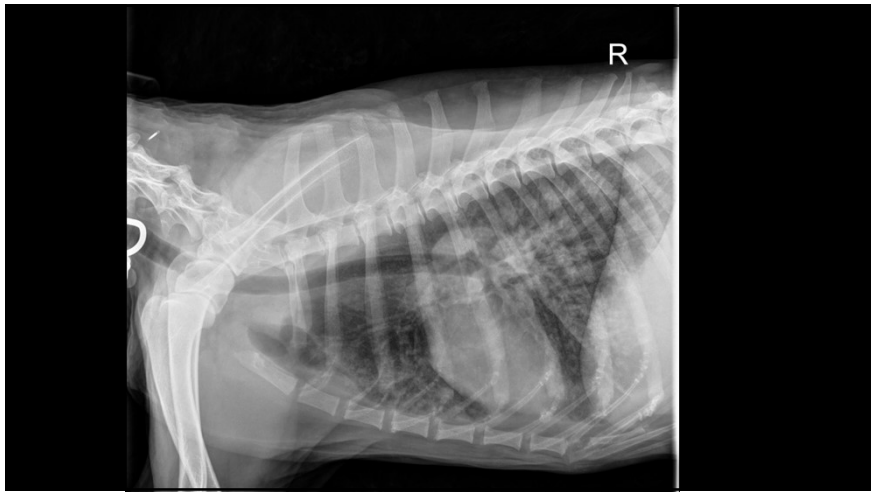

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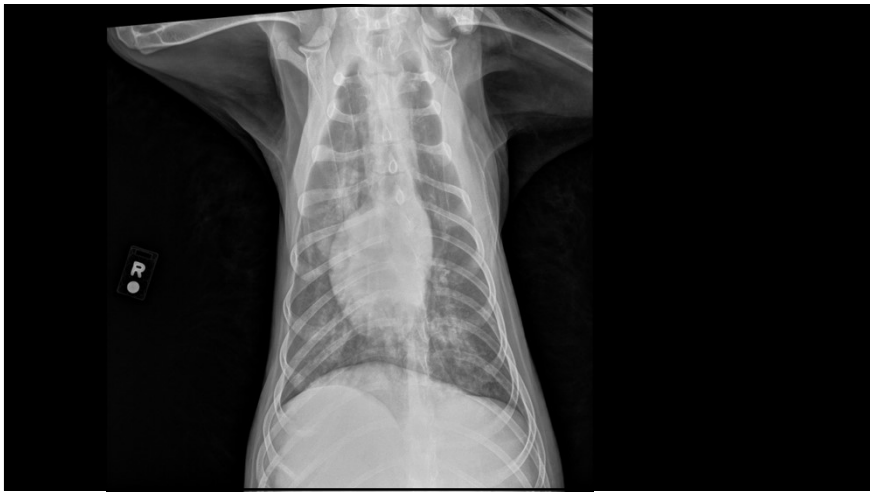

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Abdominal Ultrasound

- In some ways more worthwhile than Thoracic Radiographs
- With advent of many imaging interpretation diagnostic services this has become more commonly done in practice
- In addition to localizing Lymphoma, it could find concurrent issues
- Splenic Masses
- Mesenteric Masses
- Bladder Tumors
- Hepatic Tumors

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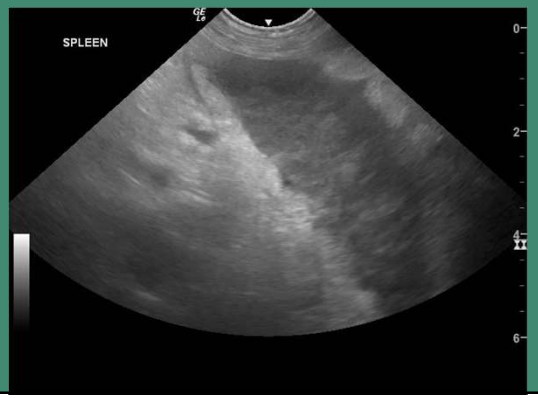
Abdominal Ultrasound



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Bone Marrow Aspirate

- Was a very common staging test until about 15 years ago.
- Performed to look for circulating Lymphoblasts in the Bone Marrow
- Or as a diagnostic test looking for Lymphoma and Leukemia
- Useful in cases of IMA and ITP
- Historically I used to perform them on all my patients with Lymphoma.
- On many dogs, would pull the sample from their pelvis with a just a local block
- Alternatively if you take the sample from the humerus you need a heavy sedation
- Spoiler Alert – I never perform them anymore!

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Immunophenotyping

- Or is Lymphoma from B Cell Or T cell Origin?
- May be the most useful Lymphoma Staging Test out there.
- Multiple ways to get this information:
  - Flow Cytometry
  - Polymerase Chain Reaction (PCR)
    (also known as PARR)
    PCR for Antigen Receptor Rearrangement
- Cytochemistry
  - Immunohistochemistry
  - Immunocytochemistry

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Flow Cytometry and PARR

- Many advances in cellular testing over the past few years, but most clinically useful has been Flow Cytometry and PARR
- Both tests are relatively affordable and very easy to perform
- Non-invasive – often just an aspirate or blood sample
- Less expensive than traditional diagnostics
  - Often replacing biopsy and bone marrow evaluation
- Added prognostic information
- Guide treatment options –
  - Monoclonal antibodies
  - cancer vaccines

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Flow Cytometry

- Primary method for subtyping lymphomas and leukemias
- Elegant way to analyze individual live cells in a suspension
- Used as a way to immunophenotype and assess clonality
- Used on thousands of cells in a sample
- Predominantly used in lymphocytic suspensions to diagnosis and characterize lymphomas and leukemias
  - Most commonly lymph nodes, blood, and spleen
  - Also liver and bone marrow
- Very useful tool in supporting a cytology suspicious of lymphoma or leukemia
- Less expensive and less invasive than histopathology

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Methods of Flow Cytometry

- Cells pass though lasers of specific and known wavelengths
- Scatter of light emitted by individual cells that fluoresce, or cells labeled with fluorescent antibodies
- Cells are filtered single file in fluid medium
- Light scatter and emission are recorded for each cell
- Results are plotted on graphs, and clusters analyzed
- Essentially capturing both the size and the complexity of the cells

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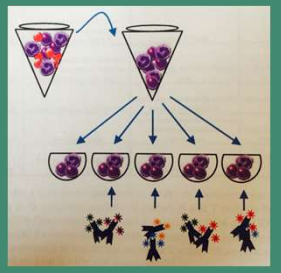
Methods of Flow Cytometry

- Always send a fluid sample
- When cells arrive at a lab, they are suspended in fluid.
- RBCS are removed
- Once RBCS removed then the sample is split up and antibodies are added. These are cellular tags. The antibodies are to common cell surface markers, and conjugated to fluorescent dyes



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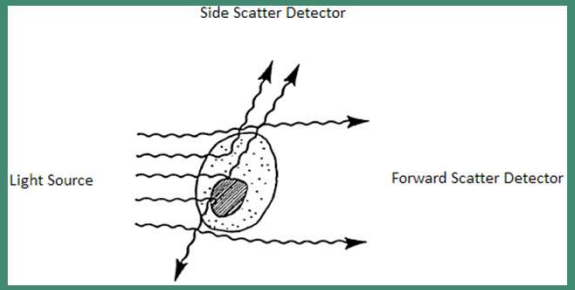
Methods of Flow Cytometry



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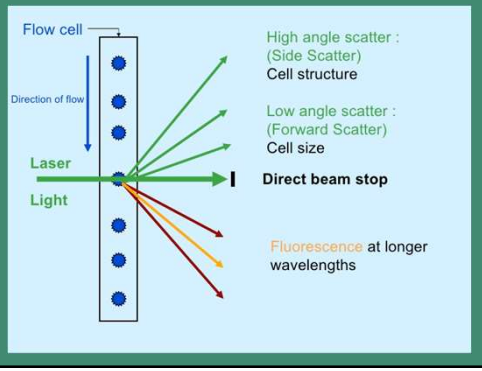
Methods of FC



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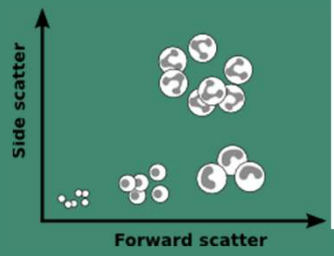
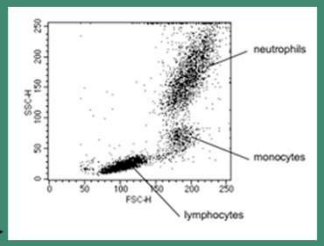
Methods of FC





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Common Antigens used for canine flow cytometry

Antigen	Expression on Canine Cells
CD_3	Mature T cells, NK cells
CD_5	Mature T cells
CD_4	T helper cells, Neutrophils
CD8	Cytotoxic T cells
CD_{21}	B cells (high levels) T cells (low levels)
CD_{22}	Mature B cells, Monocytes
CD_{34}	Leukocyte precursor (myeloid and lymphoid)
CD_{45}	All Leukocytes

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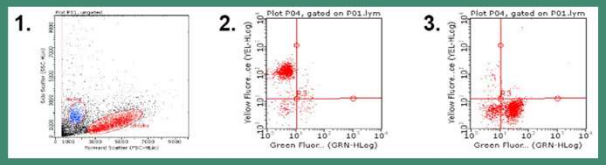
Lymphoid Leukemia Example



- 1 Ungated population of blood cells
  - Plotted via Size (FS) vs Complexity/Granularity (SS)
  - Red = Lymphocytes
  - Blue = Neutrophils
- 2. CD_4 on Y axis, CD8 on X axis (most cells express CD_4)
- 3. CD21 on Y axis, CD_3 on X axis(most cells express CD_3)
- DIAGNOSIS = T cell Leukemia

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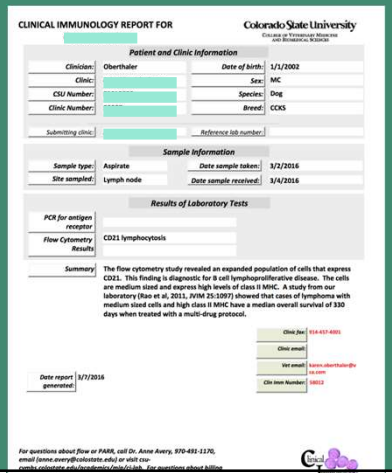

CLINICAL IMMUNOLOGY REPORT FOR

Colorado State University

Patient and Clinic Information

Clinician:	Oberthaler	Date of birth:	1/1/2002
Clinic:		Sex:	MC
CSU Number:		Species:	Dog
Clinic Number:		Breed:	CCKS
Submitting clinic:		Reference lab number:	

Sample Information

Sample type:	Aspirate	Date sample taken:	3/2/2016
Site sampled:	Lymph node	Date sample received:	3/4/2016

Results of Laboratory Tests

PCR for antigen receptor	
Flow Cytometry Results	CD21 lymphocytosis
Summary	The flow cytometry study revealed an expanded population of cells that express CD21. This finding is diagnostic for B cell lymphoproliferative disease. The cells are medium sized and express high levels of class II MHC. A study from our laboratory (Rao et al, 2011, JVIM 25:1097) showed that cases of lymphoma with medium sized cells and high class II MHC have a median overall survival of 330 days when treated with a multi-drug protocol.

Date report generated: 3/7/2016

Clinic fax: 614-457-4001

Clinic email:

Vet email: karen.oberthaler@vs.com

Clin Imm Number: 18012

For questions about flow or PARR, call Dr. Anne Avery, 970-491-1170, email (anne.avery@colostate.edu) or visit csu-cvmbs.colostate.edu/academics/mip/ci-lab. For questions about billing

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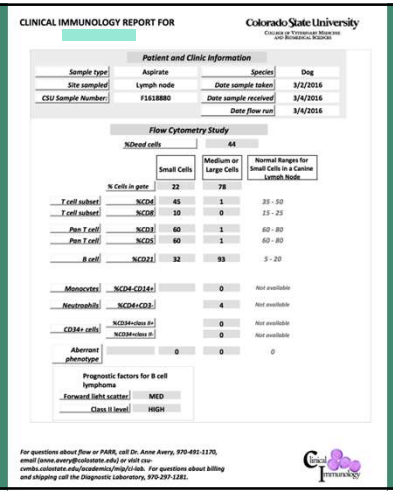

CLINICAL IMMUNOLOGY REPORT FOR

Colorado State University

Patient and Clinic Information

Sample type	Aspirate	Species	Dog
Site sampled	Lymph node	Date sample taken	3/2/2016
CSU Sample Number:	F1618880	Date sample received	3/4/2016
		Date flow run	3/4/2016

Flow Cytometry Study

%Dead cells 44

		Small Cells	Medium or Large Cells	Normal Ranges for Small Cells in a Canine Lymph Node
	% Cells in gate	22	78	
T cell subset	%CD4	45	1	35 - 50
T cell subset	%CD8	10	0	15 - 25
Pan T cell	%CD3	60	1	60 - 80
Pan T cell	%CD5	60	1	60 - 80
B cell	%CD21	32	93	5 - 20
Monocytes	%CD4-CD14+		0	Not available
Neutrophils	%CD4+CD3-		4	Not available
CD34+ cells	%CD34+class II+		0	Not available
	%CD34+class II-		0	Not available
Aberrant phenotype		0	0	0

Prognostic factors for B cell lymphoma

Forward light scatter	MED
Class II level	HIGH

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

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Practical Tips for FC

- Must be liquid sample
  - Send blood (in lavender top tube) or suspend cells in saline
  - To make a cell suspension:
    - Have a red top tube with about 1ml saline
    - Aspirate node
    - Expel the aspirate sample into the tube of saline until it's cloudy – about 2-4 aspirates worth
    - Add 0.1ml serum (ideally from patient but doesn't have to be)

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Practical Tips for FC

- Must be LIVE cells
  - Always overnight delivery
  - Always use ice
  - "Never "add-on" to the original specimen at the lab. Always send a new sample
  - Add slides for backup
    - PARR
    - Tape the slide containers to the tube, so the tube doesn't get lost in unpacking
- I use CSU (but also labs at NCSU, MSU, KSU has too.)

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PARR

- PARR = PCR for Antigen Receptor Rearrangements
- PCR = Polymerase Chain Reaction
- A PCR assay used to amplify DNA of a cell sample
- Tests for clonality
  - Single clone = Neoplasia
  - Multiple clones = Reactive process

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PARR

- Cells are derived from a variety of sources (blood, cavity fluid, or scraping off a cytology slide)
- DNA is isolated using a commercial kit
- DNA is added to PCR reaction tube with Taq polymerase and fluorescently labeled primers
- Final product is millions of copies of genes become amplified, all of which are fluorescently labeled
- Analyzed by capillary gel electrophoresis

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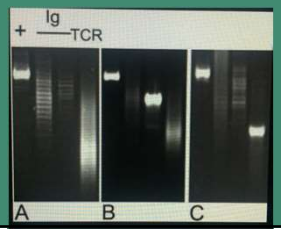
PARR

- Load them on a capillary gel, where they are separated by size.
- As the products pass through the capillary gel, a laser excites the fluorescent molecule and the emission is detected by an instrument.
- An images generated showing the different products lined up by size



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PARR

- Can be used to
  - Determine is LSA is present,
  - Immunophenotype the disease (amplifies both B and T cells)
  - Look for minimal residual disease during treatment
- In dogs PARR has sensitivity and specificity of ~90%
- Can run on dead cells, LN aspirates, bone marrow aspirates, cellular effusions, blood, cytology slides, fresh frozen tissues
- Can run on stained slides
- **Cannot always** run on formalin fixed tissue. Check with the lab!

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Flow Cytometry and PARR

- Some of the most useful tests available
- Noninvasive
- Affordable

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Flow and PARR Poll

INSERT POLL QUESTION HERE:
How many folks routinely use Flow and PARR?
a) I use both of them a lot
b) I recommend them, but clients rarely agree to them
c) I've used Flow more than PARR
d) I've used PARR more than Flow
e) Never used them before!

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Special Stains

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Special Stains

- Histochemical vs Immunohistochemical
  - Used for poorly differentiated tumors
- Histochemistry
- - chemicals when applied to tissue section have a direct reaction with tissue components
  - Technically H & E is a histochemical stain
  - Most common:
  - Toluidine blue and Giemsa - canine MCT granules
  - Periodic Acid Schiff PAS – feline (and ferret) MCT granules
  - Silver Stains like Pascuals – can r/o neuroendocrine tumors
  - Sudan Black and Oil Red O will stain lipid – useful for liposarcomas
  - Melanin bleach or Iron Stain (Prussian Blue) can tell between melanin and hemosiderin
- Most important point about histochemical stains – FREE!

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Immunohistochemistry

- Is now an integral part of routine tumor diagnostics
  - Drastically changed in the past 10 years
- Refers to the process of detecting antigens (proteins) in cells by exploiting the principles of antibodies binding to specific antigens
- Immuno – refers to using antibodies to tag the antigens
- The antibodies are conjugated to:
  - an enzyme, such as perioxidase, that can catalyze a color producing reaction
  - Or a flourophore such as fluorescein

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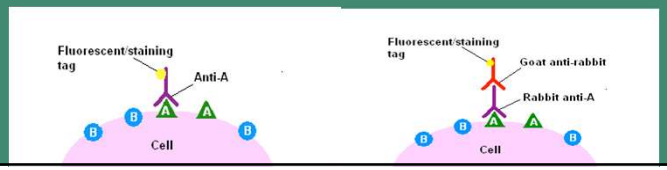
Immunohistochemistry

- Use formalin fixed tissue
  - Unstained slides
- Direct method – one step. Labeled antibody reacts directly to the target antigen
- Indirect method – unlabeled primary antibody, that binds to target antigen and a labeled secondary antibody, that reacts with primary (much more sensitive reaction



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Immunohistochemistry

- Adequate fixation is essential
  - 10 % neutral buffered formalin
  - Tumor sample less than 2 cm thick
    - Large tumors (margin evaluation) should have a few partial slices
    - Ratio of fixative and tumor is at least 10 to 1
    - Samples should be fixed 24-48 hours. Labs don't work 7 days a week.
- Lots of reasons for a negative stain – processing, anaplastic tumor, necrosis, autolysis, hemorrhage, etc.

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Immunohistochemistry

- Some stains are more popular than others
- Often suggested by the pathologist:
- Most common stains

Tumor	Stains
Lymphoma	CD_3 (T cell), CD79a (B cell)
Sarcoma	Vimentin +, cytokeratin -
Carcinoma	Vimentin -, cytokeratin +
GIST	Ckit +
Melanocytic tumor	Melan A
MCT	C-kit, usually done by histochemical stain
Histiocytic Sarcoma	CD18+, CD_3-, CD79a-
HSA	Factor VIII +

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Immunohistochemistry

- Remember must be used in conjunction with histology
- Stains do not differentiate between malignant and benign
- Ideally by same pathologist

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Immunocytochemistry

- A less commonly used staining
- The methodology is similar to immunohistochemistry
- Benefits are that you can add it on to a slide
  - Wright Geimsa stained slides are okay
- More limited number of stains
- LSA is most common:
  - CD_3 – T cells
  - PAX_5 – B cells
  - CD18 – Pan leukocytes/neutrophils

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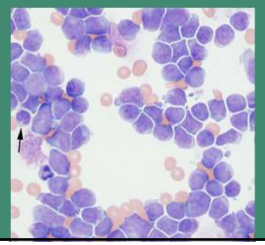
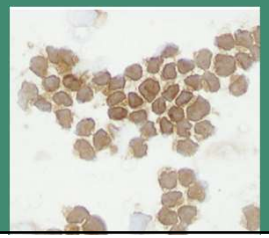
Immunocytochemistry

- Similar mechanism as IHC
- Limited by quality of the slides
- Always needs to be interpreted with cytology

Canine lymph node



Canine lymph node stained with Pax-5 (B-cell) and CD_3 (T cell). T cell lymphoma.



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"Stage Migration"

A term referring to the 'improved' survival of patients with cancer

- by either reclassifying them into different prognostic groups,
- recognizing more subtle disease manifestations,
- or by diagnosing the disease at an earlier stage.

It will result in an improved prognosis, without truly improving an individual's survival time.

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J Vet Intern Med 2007;21:1041–1047

Stage Migration in Dogs with Lymphoma

Andrea B. Flory, Kenneth M. Rassnick, Tracy Stokol, Peter V. Scrivani, and Hollis N. Erb

Background: Various diagnostic tests have been used to assign a clinical stage to dogs with lymphoma. As more sensitive staging methods are introduced, dogs are reclassified as having a higher disease stage, thereby affecting comparisons of dogs across differently staged clinical trials, and possibly, prognosis.

Hypothesis: The addition of more sensitive staging tests causes stage migration in dogs with lymphoma.

Animals: Fifty-nine client-owned dogs with previously untreated cytologically or histologically confirmed lymphoma

Methods: For every dog, the World Health Organization stage classification (I–V) was based on 5 groupings of various diagnostic tests: A (physical examination [PE] and quantitative blood count [QBC]), B (PE, QBC, thoracic and abdominal radiographs), C (PE, complete blood count with blood-smear evaluation [CBC], thoracic and abdominal radiographs), D (PE, CBC, thoracic radiographs, abdominal ultrasound), and E (PE, CBC, thoracic radiographs, abdominal ultrasound, and bone-marrow cytology). Dogs were treated with doxorubicin-based protocols.

Results: There was migration between all of the staging methods except D to E. However, the stage was not a predictor of remission rate, remission duration, or survival, regardless of staging method used.

Conclusions and Clinical Importance: These data emphasized the need for standardized methods to determine the clinical stage in dogs with lymphoma.

Key words: Cancer; Canine; Diagnostics; Prognosis; Staging.

The goals of staging dogs with cancer are to determine the optimal choice of therapy, monitor response to therapy, impart an accurate prognosis to owners, and stratify dogs for clinical trials.[1] At least 11 methods to determine World Health Organization (WHO) for canine multicentric lymphoma stage have

to longer survival times compared with historical control populations in which detection was synchronous with clinical disease and which thus has an inherently shorter survival time.[1]

The combination of the taxonomic and statistical

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"Stage Migration"

Flory et. al. found that there was no association between WHO stage and remission rate, remission duration, or survival, regardless of the staging method that was used.

The results of this study show that most dogs have liver and splenic involvement of their Lymphoma, and we know that now because we look for it.

The average prognosis for Lymphoma has not changed, while our staging tests will repeatably place dogs in higher WHO stages.

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So are these tests really used?

- My patients are rarely fully staged any more.
- I do discuss all the testing
- However, I explain that often dogs have Lymphoma in their abdominal cavity and therefore staging just to put a "WHO Stage" label to their pet's disease isn't useful
- I pick and chose the tests
- Finances are always taken into consideration
- And of course general labwork is always done if they plan on treating with chemotherapy

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Which is most useful

INSERT POLL QUESTION HERE:

Which staging tests give a family more prognostic information about their pet?

a) Immunophenotyping tests (B vs T)
b) Thoracic Radiographs and Ultrasound

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Conclusion

- No, most dogs in practice are not fully staged anymore!
- Bone marrows evaluations have fallen way down the list of performed diagnostics
- Immunophenotyping tests (Flow Cytometry, PARR) have moved up in importance and use
- Chest radiographs and abdominal ultrasounds are nice to have as baseline, but do not *generally* affect prognosis or choice of treatment protocol.
- In an ideal world would we like everything – absolutely! But truly not needed.

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Questions?

Remember to **download the CE certificate** in the handouts panel of the webinar control panel.
NOTE: CE certificate not available for watching the recording.

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Thank you for joining us!

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