Designing and Publishing Observational Studies in Veterinary Pathology

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Veterinary Pathology
Further reading...


Editorial: Ensuring observational studies have validity and impact. *Veterinary Pathology*, November 2018.
Definitions

Experimental study: infect cats with virus vs placebo and study the outcome of RCM

Observational study: measure prevalence of virus infection in natural cases of RCM

Descriptive study: describe findings in a series of cats with RCM, including prevalence of virus infection

Analytic study: compare prevalence of virus infection in cases of RCM vs normal

"Feline panleukopenia virus is not associated with …restrictive cardiomyopathy in cats"

Exposure/event → outcome

virus infection → restrictive cardiomyopathy

restrictive cardiomyopathy → serum troponin levels
Overview

- Study design and use of a specific hypothesis/objective
- Descriptive vs analytic studies
- Sequence of causation
- Numbers of study subjects
- Defining and selecting the study groups
- Bias and confounding
- Precise and objective diagnostic and grading criteria
- Approaches to increased rigor
- Validation of methods
How to create important new knowledge

• Identify important problems, work toward solutions

• Innovative mindset
  Actively search for new possibilities.
  Probe observations that don’t fit existing knowledge.
  Investigate alternative interpretations of existing data.

• Apply new methods to existing problems, if they might lead to new knowledge with impact.
Focus the study on a Hypothesis/ Question/ Objective

- Specific & precise
- Testable, definitively answerable

- Do not start with methods, they are just means to an end
Rare sightings of hypotheses in *Veterinary Pathology*

- Chronic glaucoma in dogs: relationships between histologic lesions and the gonioscopic diagnosis of pectinate ligament dysplasia.
  
  “We hypothesized that the histologic diagnosis of PLD [does] not correlate with the gonioscopic diagnosis of PLD, and that PLD cannot be diagnosed solely by routine histological examination in canine globes affected with chronic glaucoma.”

- Parvovirus infection is associated with myocarditis and myocardial fibrosis in young dogs.
  
  “We evaluated the hypothesis that myocardial CPV-2 infection is … associated with cardiac damage in dogs less than 2 years old.”
When should we form the hypothesis/ question/ objectives?

a) Before the study begins
b) When we first see trends in the data
c) After the data are analyzed
d) While writing the paper
Key point #1: use the scientific method, and a specific hypothesis/question/objective

Rationale: framed by the existing state of knowledge, what is the gap in knowledge?
Descriptive studies: the foundation of veterinary pathology

- Near the bottom of the “hierarchy of evidence”
- The basis for much of our knowledge of veterinary pathology
- Aim for multiple cases to properly document the range of findings
Number of articles published per year, by article type

- Green bars: Analytical
- Blue bars: Descriptive
- Red bars: Experimental

Year of publication:
- 2012
- 2013
- 2014
- 2015
- 2016
- 2017
Some highly downloaded & cited descriptive papers

- Salient lesions in domestic ruminants infected with the emerging so-called Schmallenberg virus in Germany
- Pathology of *Clostridium perfringens* type C enterotoxemia in horses
- Initial case reports of cancer in naked mole-rats
3 ways to improve this study of published papers?
Average number of citations per article per year

Year of publication

Average number of citations per article (with 95% confidence interval)

Year of publication

Analytical
Descriptive
Experimental
Descriptive vs Analytic Studies

If your objective is to “describe”, try changing it to “compare” for a more powerful study design.

A simple step to greater insights.
Analytic studies in *Veterinary Pathology*

- Canine lymphomas: association of classification type, disease stage, tumor subtype, mitotic rate, and treatment with survival
  
  histologic type $\rightarrow$ survival

- Histomorphometry of feline chronic kidney disease and correlation with markers of renal dysfunction
  
  $\pm$ renal fibrosis $\rightarrow$ serum creatinine level

- EcPV2 DNA in equine papillomas and squamous cell carcinomas...
  
  $\pm$ papillomavirus $\rightarrow$ $\pm$ squamous cell carcinoma
Key point #2. Consider which study design best addresses the study objectives.

Descriptive   Analytic   Experimental
Direction of causality?
A limitation of analyzing postmortem material

Equine multinodular pulmonary fibrosis: a newly recognized herpesvirus-associated fibrotic lung disease

EHV-5 → EMPF

OR

EMPF → ↑ detection of EHV-5
Direction of causality?
A limitation of analyzing postmortem material

Valvular and mural endocardiosis in aging zebrafish:
-56% of fish with ‘smoothened’ mutation
-10% of wild-type fish

Endocrine pancreas in cats with diabetes mellitus
Islet T & B cells \(\rightarrow\) islet damage \(\rightarrow\) diabetes

OR
Islet damage \(\rightarrow\) islet T & B cells \(\rightarrow\) diabetes
“These findings suggest that interleukin-8 plays an important role in pneumonic pasteurellosis.”

Morphology vs function

In situ hybridization

H&E

Antisense

In situ hybridization

PAMPs ↔ IL-8 → Neutrophil recruitment

GRO-α ↔ C5a
Key point #3.
Consider if the sequence of causation is important,
and whether it can be addressed by the study design.
It’s all in the numbers…

• A frequent limitation of studies in *Vet Pathol*

• **Number of cases and controls**: what is needed to definitively test the hypothesis or answer the question? *(Sample size calculation)*

• **Ratio of controls to cases**
  - 1:1, if cases are frequent
  - up to 3:1, if cases are rare
  • No advantage of having fewer controls than cases
Beat the odds! Adding cases from another institution

- SP-A and napsin A in the immunohistochemical characterization of canine pulmonary carcinomas: comparison with TTF-1

- Histologic and IHC characterization of pheochromocytomas in 20 clouded leopards

- WSAVA Renal Pathology Initiative: classification of glomerular diseases in dogs
Key point #4.
Ensure the number of study subjects is adequate to meet the objectives
Consideration of the study population: special considerations for laboratory case material

- General population vs study population
  Eg. Differences in mortality rate, antibiotic therapy

- Do controls effectively match the cases?
  Eg. same population, geographic origin, quality of veterinary care, prevalence of infectious agents, nutritional status, concurrent diseases, sample quality
  … might these explain the observed differences?

- Are we measuring new (incident) or existing (prevalent) cases?
Selecting cases and controls

• Inclusion & exclusion criteria: cases and controls
• Objective, explicit, reproducible diagnostic criteria
• Controls: more important than the cases?
  – What is the best control, to address the study objectives?
  – Do the controls match the cases?
• Sampling a subset?

The iterative process of selecting cases and controls

• Same sample population
• Comparable inclusion and exclusion criteria
• Identical to cases in all ways except the disease?
Effective selection of cases and controls

- X-linked hereditary nephropathy in Navasota dogs: clinical pathology, morphology, and gene expression during disease progression. **Controls are sex-matched littermates unaffected by the disease.**

- Evidence of the primary afferent tracts undergoing neurodegeneration in horses with equine degenerative myeloencephalopathy based on calretinin immunohistochemical localization. **Controls age-matched to cases, and both normal and "other spinal disease" controls are both essential for the study outcome.**
Effective selection of cases and controls

Feline panleukopenia virus is not associated with myocarditis or endomyocardial restrictive cardiomyopathy in cats.

A search of the archives between June 2007 and November 2014 was performed [method of selection], and cases limited to cats at least 1 year of age were identified using the keywords feline or cat and endomyocardial fibrosis, endocardial fibrosis, endocardial scar, endomyocarditis, or restrictive cardiomyopathy [inclusion criteria]. We excluded cases having keywords hypertrophic and dilated [exclusion criteria]. Control cases were identified using keywords describing acute trauma, neoplasia, or other noncardiac causes of sudden death [inclusion criteria for controls!]. A similar age distribution of control cases was selected from the same time period and source [matching of controls to cases].
Key point #5.
Clear and objective definition of the study groups (cases and controls) is essential to the validity of any observational study.
Sources of bias

Are the cases and controls similar, except for the presence/absence of the disease?

• Method of acquiring subjects
• Demographic details
• Availability of data
• Sample quality

• Methods/ assessments
• Clinical case management
• Likelihood of survival/ death
• Loss from follow-up

… if not, will the differences bias the results?
… and what can be done about it?
Measure alternative causes, confounding factors, and sources of bias

(Alternative causal factor)

Age

(Event/exposure)

Nodal mets detected

↑ Survival

(Outcome)

Referral centre (vs primary care)

(Selection/misclassification bias)

Unmeasured factors might plausibly affect the outcome?

• Measure these factors for cases and controls
• Assess their frequency, in a data table
• Control by analysis, exclusion, or matching
• Also consider dose/timing of the exposure
Key point #6.
The concepts of bias and confounding:

- Unfamiliar to most pathologists
- Of major concern for studies based on archived case material
- Essential to the validity of observational studies
1. Will the study design definitively address the hypothesis/question/objective?

2. Are the number of cases and controls adequate, for the expected variability of the data?

3. Is the study coherent and focused on a hypothesis/question/objective?

4. Would additional analyses add value to the findings?
Why have we placed methods near the end of this seminar?

Hint:

- “Create a hypothesis/question/objective…”
- “Don’t start with the methods; they are only a means to an end”
Objective & explicit diagnostic criteria

Bovine papilloma-virus DNA and S100 profiles in sarcoids and other cutaneous spindle cell tumors in horses. (Epperson et al, 2017)

**Sarcoid.** Tumors classified as classical sarcoids were poorly demarcated, infiltrative, unencapsulated dermal masses composed of spindle-shaped fibroblasts with indistinct cellular borders and a scant to small amount of eosinophilic cytoplasm. Spindle cells were arranged in a storiform, herringbone, and/or whorled pattern and were closely associated with the epidermis. Nuclear palisade patterns (ie, Antoni A patterns) were occasionally present in tumors classified as sarcoid for other characteristics (eg, close epidermal association with rete pegs and acanthosis). Epidermal changes included acanthosis and prominent epidermal rete pegs. Presence of a “picket fence” perpendicular orientation of superficial dermal fibroblasts to the basal epidermis, decreased density of adnexa, and variable ulceration were also defining criteria for a diagnosis of sarcoid. Mitotic count was variable but was generally low, averaging no more than 3 to 4 mitotic figures in ten 400× fields (field of view, 0.196 mm²). There were several sarcoids with the nodular subtype of sarcoid that were characterized by an infiltrative deep dermal or subcuticular, poorly to well-demarcated nodular mass of spindle-shaped fibroblasts with indistinct cellular borders arranged in a storiform pattern similar to that of classic sarcoid. Involvement of the overlying dermis and epidermis was minimal.

**PNST.** For diagnostic categorization, tumors of peripheral nerve sheath origin (ie, so-called malignant/benign PNST, schwannoma, neurofibroma, neurofibrosarcoma) were grouped together. These neoplasms minimally comprised spindle cells with indistinct cellular borders arranged in a whorled, herringbone, and/or storiform pattern that exhibited concentric lamination centered on nerves or loose bundles of connective tissue that resembled nerves. Some of these tumors were also variably characterized by densely cellular Antoni A areas that had scant collagenous stroma, with short fascicles of cells with palisading nuclei and Verocay bodies. Antoni A areas were occasionally admixed with hypocellular Antoni B areas, which had abundant myxoid stroma. Less commonly observed features of nerve sheath origin neoplasms included hyalinization of vascular walls and stroma, tumor encapsulation, and irregular cytoplasmic vacuolation of neoplastic cells.
Grading schemes cannot be validly used if the grading criteria are imprecise or subjective.

Table 4. Interobserver Agreement Between 3 Evaluators Assigning Histologic Grades to Canine Appendicular Osteosarcoma Tumors Using 2 Different 3-Tier Grading Systems, Including Artificial Conversion to 2-Tier.

<table>
<thead>
<tr>
<th>Histologic Grade</th>
<th>2002 System(^a)</th>
<th>2007 System(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Agreement (%)</td>
<td>Fleiss's Kappa</td>
</tr>
<tr>
<td>I–III</td>
<td>19</td>
<td>-0.0129</td>
</tr>
<tr>
<td>High (II–III) vs low (I)</td>
<td>42</td>
<td>-0.0117</td>
</tr>
<tr>
<td>High (III) vs low (I–II)</td>
<td>66</td>
<td>0.1030</td>
</tr>
</tbody>
</table>

\(^a\) Kirpensteijn et al.\(^1^8\)

\(^b\) Loukopoulos and Robinson.\(^2^2\)

“No correlations were observed between grade and either median survival time or time to development of metastatic disease.”

Key point #7.
Diagnostic and grading criteria must be precise & objective, if the study results are to be implemented by others.
A passion for the truth: increasing rigor in veterinary pathology studies

• Independent replication of findings
  (eg. confirmation on different samples, analyzed on a different day)

• Redundancy: ≥2 methodologies for important findings. Eg. RT-qPCR and IHC.

• Test findings on a 2nd population of animals
  – Validation of important cancer grading schemes
  – By the same or by independent investigators
Cytologic criteria for mast cell tumor grading in dogs with evaluation of clinical outcome.
• Independent grading by 3 anatomic pathologists and 3 clinical pathologists.

Two canine papillomaviruses associated with metastatic squamous cell carcinoma in two related basenji dogs.
• Redundant methods--IHC, PCR, ISH--to validate the findings.

Prognostic significance of canine mammary tumor histologic subtypes: an observational cohort study of 229 cases.
• Validation of an already-published grading scheme in a new population
Quantitative analysis, blinding, statistical analysis

- Quantitative assessment
- Systematic methodology
- Clear definitions of diagnoses
- Blinding, especially for subjective analyses
  - Randomized, vs separated cases and controls
- Stats: seek professional help, for confidence in the analysis
Validation of methods

1. Validate methods before use in the study
2. Quality control when analyzing study materials
   - Biologic cases & controls
   - Internal positive and negative controls
   - Technical negative & positive controls
     No-antibody vs irrelevant antibody
   - **Validation of antibody specificity**
Bovine Pneumonia, antibody to MDA, 1:100

Bovine pneumonia, normal rabbit serum, 1:100

Normal bovine lung, antibody to MDA, 1:100

IHC for malondialdehyde (MDA) (a marker of oxidative injury)

Credits: Courtney Schott
As the study progresses, would additional analyses add value?

A mind open to discovery

• The goal of an investigation is not only to confirm what we know, but to discover new things as the study progresses

Critical analysis

• Think deeply on alternative interpretations of existing data, and strategies to evaluate them

Added value

• After analysis of initial findings, what elements could be added to give more value or impact?
Key points

- Focus on a specific hypothesis/objective
- Merits of descriptive vs analytic studies
- Consider the sequence of causation
- Numbers of study subjects
- Defining the study groups: inclusion/exclusion criteria, appropriate controls
- Bias and confounding: differences between study groups, alternative explanations of study outcomes
- Precise and objective diagnostic and grading criteria
- Validation of methods
- Approaches to increased rigor
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