Designing and Publishing Observational Studies in Veterinary Pathology

Jeff Caswell & Andrea Gröne
Veterinary Pathology
Overview

- General considerations
- First steps
- Study design
- Selection of study subjects
- Critique of the design
- Methods

Checklist: STROBE-vet & others: Equator/Meridian
Strengthening the Reporting of Observational Studies in Epidemiology
Definitions

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

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 vs normal cats

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

Exposure/event → outcome
- virus infection → restrictive cardiomyopathy
- restrictive cardiomyopathy → protein expression
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
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
Analytic observational studies: Why not?

- Descriptive studies have no control group
- Analytic studies compare one group to another
- Improved validity of findings
- Test hypotheses
- Test causality

Adding a meaningful control group is the easiest way to take observational studies to a higher level, by allowing comparisons instead of simple descriptions.
3 ways to improve these data?
3 ways to improve these data?

Descriptive

Analytical
Overview

- General considerations
- First steps
- Study design
- Selection of study subjects
- Critique of the design
- Methods
Ensure your study has **impact** and will be **published**

Manuscripts considered for publication must:
1. have significant importance to animal and/or human health,
2. include new knowledge supported by valid data,
3. address disease mechanisms (pathogenesis, pathophysiology), or pathologic findings in important new or emerging diseases, or clinico-pathologic correlations, AND
4. be of sufficiently broad interest to be of substantial value to veterinary pathologists.
Ensure your study has **impact** and will be **published**

The secrets of success:

- Work on something important
- Take an innovative approach
- Use valid study design & methods
- Find something new
- Communicate findings effectively

Identify important problems, and work toward solutions for them

Think of the unique aspects of your caseload or situation, and the opportunities these create
How to create important new knowledge

• Work on important problems

• 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.

• Use the scientific method:

  Observation → questions → hypothesis → observational studies → critical analysis & inference → (communicate findings) → refine and repeat
Some novel, useful & important analytic studies

- 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
  
  renal fibrosis $\rightarrow$ serum creatinine

- EcPV2 DNA in equine papillomas and squamous cell carcinomas... papillomavirus $\rightarrow$ squamous cell carcinoma
Ask, at each stage of the study:

1. Is it a useful contribution to new knowledge (& what can be done now to give additional value)?

2. What critiques would reviewers make (& what can be done now to mitigate them)?

3. Will the plan **conclusively** address the hypothesis/ question/ objectives (& what can be done now to ensure it does)?
Creating the hypothesis/ question/ objective

• **Dare** to create 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

a) Form the hypothesis/question → build study design based on hypothesis → selection of cases/controls and methods are slaves of the hypothesis/question

b-d) exploratory studies: fit hypothesis to findings
Write a coherent proposal and ask others for critique

Rationale: framed by the existing state of knowledge, what is the gap in knowledge?
Bovine papillomavirus DNA and S100 profiles in sarcoids and other cutaneous spindle cell tumors in horses.

“difficulty in accurately diagnosing nodular sarcoids vs PNST”…

[has been proposed that IHC for S100 & detection of BPV DNA would differentiate these neoplasms]…

“The objective of this study was to determine whether BPV DNA tests and S100 IHC would facilitate differentiation of sarcoid from PNST”.
Overview

• General considerations
• First steps
• **Study design**
• Selection of study subjects
• Critique of the design
• Methods
If your objective is to describe, change it to compare for a more powerful analytic study.

The simplest step to greater insights.
Retrospective vs Prospective Enrolment

- **Retrospective**: easier, less expensive, quicker enrolment, more cases

- **Prospective**: standardized sampling & analysis, intentional data collection
Some design considerations that determine what inferences are possible

Method of enrolment:
- based on knowledge of: exposure (cohort), outcome (case-control) or neither (cross-sectional)

Incident (new) vs prevalent (existing) cases
- prevalence studies can’t differentiate “effects on development of disease” vs “effect of exposure on survival of diseased animals”

Was exposure measured before the outcome developed?
- longitudinal/cohort study: disease-free animals followed over time until the disease develops, necessary to show the sequence of causation.
Equine multinodular pulmonary fibrosis: a newly recognized herpesvirus-associated fibrotic lung disease

EHV-5 $\rightarrow$ EMPF

OR

EMPF $\rightarrow$ ↑ 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 → islet damage → diabetes
OR
Islet damage → islet T & B cells
   diabetes
Longitudinal sampling to infer causation in an observational study

Pathogenesis of enterococcal spondylitis caused by *Enterococcus cecorum* in broiler chickens.

Longitudinal sampling of birds to show the sequence:

1. *E. cecorum* in intestine and spleen →
2. *E. cecorum* in vertebra →
3. Osteomyelitis/spondylitis

…and that osteochondrosis can precede spondylitis (confirmed by a subsequent experimental study).
It’s all in the numbers…

• Inadequate numbers is a frequent limitation of studies in veterinary pathology

• Number of cases and controls: what is needed to definitively test the hypothesis or answer the question? (Statistical power analysis)

• 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

- Pulmonary veno-occlusive disease: a newly recognized cause of severe pulmonary hypertension in dogs
- 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
Increase the impact of pathology studies

Relate the pathologic findings to clinical outcomes

- Cytologic criteria for mast cell tumor grading in dogs with evaluation of clinical outcome
- Receptor tyrosine kinase expression profiles in canine cutaneous and subcutaneous mast cell tumors
- Histomorphometry of feline chronic kidney disease and correlation with markers of renal dysfunction

Include an analysis of causes or risk factors

- Valvular and mural endocardiosis in aging zebrafish
- Systemic amyloid A amyloidosis in island foxes: severity and risk factors
Overview

• General considerations
• First steps
• Study design
• Selection of study subjects
• Critique of the design
• Methods
Consideration of the study population:
special considerations for laboratory case material

- Written informed owner consent: is it coming?
- IACUC approval (if samples acquired for purpose of the study)
- Differences from target population, as a cause for bias  
  Eg. mortality, antibiotic therapy
- Do controls effectively match the cases? 
  Eg. geographic origin, quality of veterinary care,  
  prevalence of infectious agents, nutritional status, concurrent diseases, sample quality  
  … do 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?
  - One control, or more?
  - Do the controls match the cases?
- Sampling a subset?

The iterative process of selecting cases and controls

- Same population
- Same inclusion and exclusion criteria
- Same opportunity to develop 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].
Objective & explicit diagnostic criteria

Bovine papilloma-virus DNA and S100 profiles in sarcoids and other cutaneous spindle cell tumors in horses.

**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.
Validate the cases and controls

Study author(s) should re-examine the study materials, to avoid the variability of the original assessments

Confirm that:

• Cases are really cases; controls are not cases
• Cases and controls both meet the inclusion and exclusion criteria
• Exposures and outcomes are correctly recorded and interpreted
Overview

- General considerations
- First steps
- Study design
- Selection of study subjects
- Critique of the design
- Methods
Viciously critique your study design

• Will the study design definitively address the hypothesis/question/objective?

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

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

• Would additional analyses add value to the findings?
Unmeasured factors might plausibly affect the outcome?

- **Measure** these factors for cases and controls
- **Compare** their frequency, in a data table
- **Mitigate** by analysis, exclusion, or matching
- Also consider dose/timing of the exposure
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
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
Sources of bias

What might differ between cases and controls, and might this affect (bias) the outcome?

- Inclusion & exclusion criteria
- Other plausible causes
- Availability of data
- Sample quality
- Methods/ assessments
- Clinical case management
- Likelihood of survival/ death
- Loss from follow-up

... and what should be done about them: while designing the study, at the time of sampling, and during data analysis?
Overview

• General considerations
• First steps
• Study design
• Selection of study subjects
• Critique of the design
• Methods
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”
A passion for the truth: 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
  – Important cancer grading studies
  – Confirm exploratory studies
  – Or, later study 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 a published grading scheme in a new population
Quantitative analysis, blinding, statistical analysis

- Quantitative assessment
- Systematic methodology: necropsy data, sampling
- Clear definitions of diagnoses
- Blinding, especially for subjective analyses
  - Intermingled or separated cases and controls?
  - Blinding in the face of recognizable lesions?
- Stats: seek professional help, for confidence in the analysis
Quantitative analysis, blinding, statistical analysis

• Muscle pathology in free-ranging stranded cetaceans.
• Verminous arteritis due to *Crassicauda* sp in Cuvier's beaked whales. Standardized collection of epidemiology data, necropsy methods, BCS, evaluation of vascular system, collection of muscle samples.

• Approaches to investigating complex genetic traits in a large-scale inbred mouse aging study. Systematic sampling of routine case material allows large-scale association of pathology and genetics.

• WSAVA-RPI: Classification of Glomerular Diseases in Dogs. Meaningful statistical analysis for unbiased analysis.
Validation of methods

• **Validation of new methods**
  intracellular cytokine staining, in situ hybridization, ...

• **Technical** negative & positive controls;
  **Biologic** cases & controls

• **IHC**: no-antibody vs irrelevant antibody, Western blot for specificity,
  internal positive and negative controls.

• **RT-qPCR**: BLAST primer specificity, standards,
  RNA purity, no-template and no-RT controls, reaction efficiency,
  melting temp analysis, sequence of product, validate reference genes

• **Redundant analysis**: comparison to a different assay
We’re all individuals!
Respect for inter-observer variability

• Study authors should validate diagnoses and other laboratory data
• Documentation of inter-observer variation: kappa
• The bigger picture: does variation in how diagnostic criteria are applied poison the value of new findings in pathology?
A mind open to discovery

• The goal of an investigation is not to 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?
Meaningful addition of elements to the study

- Strain- and diet-related lesion variability in aging DBA/2, C57BL/6, and DBA/2xC57BL/6 F1 mice. Large XS descriptive study; added the genetic basis of age-related spontaneous lesions in mice.

- Spontaneous pathology and routine clinical pathology parameters in aging beagle dogs: a comparison with adolescent and young adults. XS study of background lesions; value added by determining which are different in young vs old animals.

- Identification of a unique amyloid sequence in AA amyloidosis of a pig associated with Streptococcus suis infection. Single-animal case report transformed by appropriate bioinformatics analysis and in vitro testing of amyloid fibril formation.

- Characterization of a cardiorenal-like syndrome in aged chimpanzees. Value added by quantifying and measuring the association of cardiac and renal lesions, with consideration of disease mechanisms, and relationship of clinical findings and clinical pathology data.
Key points, part 1

• Analytical studies
  – Add a meaningful comparison to any descriptive study

• Hypothesis/ Question/ Objective
  – Specific and testable/answerable
  – Methods are subservient to the hypothesis/question

• Form a clear explanation of the Rationale, framed by the existing state of knowledge and current literature, that leads obviously to the hypothesis/question/objectives

• Write a study proposal including anticipated findings, their expected significance, and the users of the findings
Key points, part 2

- Consider the elements of study design: prospective vs retrospective enrolment, incident vs prevalent cases, single vs longitudinal measurements, adequate numbers of cases and controls, and add value by relating to clinical outcomes and measuring causes or risk factors.

- Clear inclusion and exclusion criteria;
  - Objective, explicit and reproducible diagnostic criteria

- Careful consideration of the most appropriate controls

- Early consideration of alternative causes of study outcomes, confounding factors, and source of bias

- Methods to increase rigor: systematic methodology, measure inter-observer variation, validation of new methods, redundant analyses, blinding, quantitative measurements, professional statistical analysis

- Value-added: a discovery mindset, critical analysis, follow-up with added elements that contribute value