

Training for OIE National Focal Points for Wildlife

4th Cycle - Workshop Wildlife Disease Outbreak Investigations



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I. WILDLIFE DISEASE SURVEILLANCE STRATEGIES

Surveillance, prevention and control of animal diseases is a worldwide challenge: Building a world, regional and national animal health system is a global public good and a priority. Our world is undergoing unprecedented changes, all of which tend to amplify the emerging and re-emerging health risks for animals and humans.

- *World Organisation for Animal Health*

Wild animal pathogen surveillance is essential to animal health management

Surveillance for wild animal pathogens is the single most important component of a national wildlife health program. Surveillance is defined as "the systematic on-going collection, collation, and analysis of information related to animal health and the timely dissemination of information to those who need to know so that action can be taken: (OIE Terrestrial Animal Health Code). It is essential to all of the other components (prevention, detection, risk analysis, management). For OIE Wildlife Focal Points, it is the activity of greatest importance because it provides all of the information the focal point needs to carry out his or her work. Only through wildlife pathogen surveillance can a country know what pathogens are present in its wild animal populations, in which geographic areas and in which host species. Surveillance is required to detect new, emerging diseases. Surveillance also can measure the proportion of animals in a population that is infected (the prevalence of disease within the population). All of this information is required to assess health risks associated with international trade or internal movement of wild animals, and to meet international obligations for disease reporting. Surveillance also requires an organized system of observation of wild animals in the field, veterinary diagnostic laboratories, information management systems and communication systems, all of which also are required when a country decides to respond to a disease outbreak and take management actions. Thus, wildlife disease surveillance contributes to the national capacity that also is required to manage urgent domestic animal health events.

The key points are:

1. That it is a continuous activity, a constant investigation and vigilance for pathogens in wildlife and the diseases they may cause;
2. That surveillance involves not just the collection of information but also the regular analysis of the data for specific purposes; and
3. That surveillance includes communication of the results of data collection and analysis to the full range of people, agencies and institutions that need the information. Thus, a surveillance programme has several different components:
 - detection of dead or diseased wild animals, or collection of samples from wild populations
 - identification of pathogens and diseases (diagnosis, laboratory tests)
 - information management: computerized records of all information
 - data analysis and communication: maps, statistics, reports, risk analysis, meetings.

The importance of surveillance of wildlife cannot be over-emphasized.

As an example, it was through general passive surveillance that a new form of Highly Pathogen

Avian Influenza (HPAI) was initially detected in the United States in December, 2014. This detection occurred in the State of Washington, and through genetic analysis was determined to be a new H5N8 Asian strain (identified as Clade 2.3.4.4). The detection was communicated to our partners nationally to provide advance warning and enhance the ability to monitor for disease in agriculture species. The same strain (with identical genetic structure) was detected in the Republic of Korea, and ultimately reported in China, Japan, Russia and Europe.

1. Forms of pathogen and disease surveillance

The many aspects of animal health surveillance are described in Chapter 1.4 of the OIE's Terrestrial Animal Health Code. However, some aspects of pathogen and disease surveillance in wild animals require special attention. In wild animal populations, probability-based sampling methods (Terrestrial Animal Health Code, Chapter 1.4.4) seldom can be used for individual animals because of practical problems of access to wild animals and lack of accurate information about population sizes and structures. However, it is possible to conduct probability based sampling of geographic areas containing wild animals when samples are being collected across a spatial extent (see Study Design section in this document). In general, most samples in wildlife pathogen surveillance will be non-random and will be based on what is possible to achieve given the difficulties of securing samples from wild populations (often called "convenience sampling"). This will affect the analytical approaches that can be applied to the surveillance data and the nature of the conclusions that can be drawn from the data, and therefore should be limited to the extent possible. Nonetheless, such surveillance remains a powerful and essential tool in national and international management of animal and human health, and should be carried out in every country.

There are two quite different forms of pathogen surveillance. One is general or scanning surveillance (sometimes called "passive: surveillance, although there is nothing "passive: about such surveillance programmes). The other form is targeted surveillance, which focuses on a particular pathogen in specified wild animal populations (sometimes also called "active: surveillance). Both forms of pathogen surveillance are important in a national wildlife health programme.

General or "scanning: wildlife pathogen surveillance is a particularly critical component of a national wildlife health programme. It is impossible to have a complete national animal health programme unless a country has a programme of general wildlife pathogen surveillance. General surveillance is the means by which a country can ascertain what pathogens exist in its wildlife, and it is the only available form of national vigilance for emerging diseases associated with wild animal pathogens.

2. Components of a general surveillance programme for wildlife pathogens

As noted earlier, wildlife pathogen surveillance consists of four very different activities which must be tightly coordinated into a cohesive surveillance programme. Each of these four components involves different people with different training and expertise and, often, from different branches of government or from non-government organizations or universities.

a. Detection of pathogens and diseases

General surveillance for wildlife pathogens and diseases most often begins with detection of sick or dead wild animals. Most general wildlife pathogen surveillance programmes are based on examination of wild animals found dead. Therefore, dead wild animals are the most important resource to the surveillance programme. Thus,

the first component of a general surveillance programme for wildlife pathogens is a network of people who are likely to encounter dead or sick, wild animals. These same people or others must be prepared and trained to collect these dead wild animals and transport them to an animal disease diagnostic laboratory, or they must be trained to necropsy such animals in the field and send the correct samples to the laboratory. Who can carry out this work? The answer to this question may differ among countries, but a successful programme will require a network of people who spend time in areas inhabited by wild animals and who are informed how to report dead or sick wildlife to authorities who are responsible for ensuring adequate specimens are sent to an appropriate laboratory. Thus, the programmes responsible for wildlife pathogen surveillance must recruit the interest and cooperation of a wide range of people who spend time in wild animal habitats. Such people include, particularly, government wildlife officers and biologists, usually associated with ministries, departments or agencies (federal, state/province, regional) responsible for wildlife management. These people require permission and encouragement from their employers to participate in the surveillance programme. Other potential participants include hunters, fishermen, naturalists, university scientists, non-government conservation organizations, wildlife rehabilitators and the general public. To obtain their participation in the surveillance programme, such people must regularly be informed about the programme, encouraged to participate and rewarded for doing so. They may require assistance, such as free telephone access to surveillance programme staff, special training sessions, and sometimes also financial assistance, and should receive reports on the results of the surveillance programme to maintain their interest and collaboration. Those responsible for the wildlife pathogen surveillance programme will need to spend time and resources every year to maintain and support this network of people engaged in detection of sick or dead wild animals and the transport of specimens to laboratories. It is important to establish leadership on biosafety and personal protective equipment and sample handling when providing assistance to surveillance staff.

b. Identification of pathogens and diseases

Once dead or diseased wild animals are detected, they must be examined to determine why they are sick or dead, and what pathogens they may carry. Who can carry out this work? This work can only be carried out by well-trained animal pathologists in appropriately equipped animal disease diagnostic laboratories, which also employ well-trained microbiologists, molecular biologists, parasitologists and toxicologists. Such laboratories must be capable of identifying a wide range of viral, bacterial, protozoal, fungal, and metazoan infectious pathogens, and also a wide range of toxins and environmental contaminants and poisons. Such laboratories most often are associated with a country's government ministry, department or agency responsible for agriculture, domestic animal health and Veterinary Services. Thus, the ministries or agencies responsible for wildlife and the ministries or agencies responsible for veterinary diagnostic laboratories usually must collaborate closely on any wildlife pathogen surveillance programme, if these functions are separated.



Opportunity to review options that exists for Wildlife Disease Surveillance Strategies

(Understanding samples and techniques that may be used for wildlife)

1. Question: What are the advantage and disadvantages of different techniques and the unique challenges concerning wildlife disease surveillance?
2. Discussion: Consider types of samples or data that can be collected: whole carcasses, live trapping, sampling of hunter-harvested animals, sampling road-killed animals, use of proxy species, environmental sampling, questionnaire surveys, syndromic surveillance, etc. Discuss benefit of each of the types, including access, cost, resources needed to obtain samples, validity, etc.
3. Question: Are there any recent examples of successful general and targeted surveillance projects that have been ongoing or recently developed?
4. Question: Can you give reasons for using certain methods for sample collection? Are there limitations on analysis based on which types of samples are collected?

Additionally, please discuss logistics with invasive (blood, organ samples) and non-invasive sampling (oral/ nasal/ cloacal/ skin swabs).

II. WILDLIFE DISEASE OUTBREAK INVESTIGATION

1. What is an outbreak?

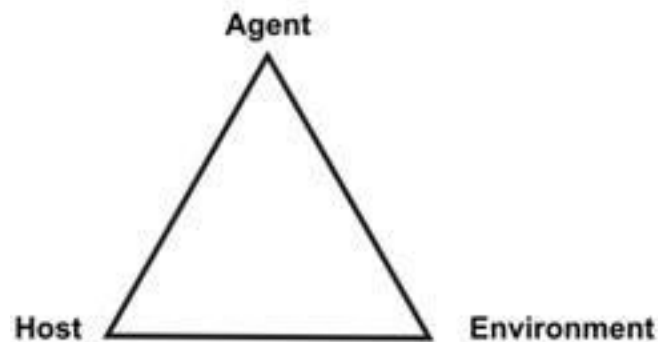
- OIE: The occurrence of one or more cases in an epidemiological unit.
- Centers for Disease Control and Prevention (CDC): An increase in the number of disease cases over and above an "expected: number per given place or time period.
- In rare diseases, one case may be considered an outbreak.
- Some reportable, highly contagious or damaging diseases may warrant an investigation by law.

Different types of outbreaks

1. Defined population, but unknown pathogen. Common point source exposure may be discovered.
2. Defined pathogen, but unknown population. Must use case-control study approach and best clues for exposure may be demographic distribution of cases.
3. Defined pathogen in a known population. You must look for the source...and subsequent exposures (traces).
4. Unknown population and pathogen. A cluster of syndromic cases within a large geographical area. These may or may not be related.

An emerging disease is defined as a new infection resulting from the evolution or change of an existing pathogen or parasite resulting in a change of host range, vector, pathogenicity or strain, or the emergence of a previously unknown pathogen. When an agent appears for the first time in a new geographic or host range, it may be considered an emerging disease. Globalization, the change in weather patterns, and increase in speed and volume of international transport as well as passenger travel are known factors that could favor the spread of pathogens and the emergence of disease.

For your review: Factors that are important to consider when investigating an emerging infectious disease are represented in the epidemiological triad:



While the remainder of this portion of the training manual will focus on preparation and response to an infectious disease outbreak, it is wise to remember that a wildlife mortality event may be caused by various types of infectious agents (viral, bacterial, fungal, parasitic or prion) or could be caused by biotoxins (algal toxins, mycotoxins, avian botulism), chemical toxins (oil, lead, mercury, cyanide, salt, organophosphate compounds, insecticides, pesticides, etc.) or by electrocution, severe weather changes, or trauma.

Planning for response: Effective planning for combating wildlife disease outbreaks requires an understanding of disease control operations and the basic needs such as personnel, equipment, supplies, and permits.

2. Outbreak investigation – Step by step

The aim of reviewing and familiarizing teams with steps in an outbreak investigation is to ensure team readiness to respond to an actual outbreak. A simulation exercise may be used in conjunction with this overview to strengthen awareness of veterinarians and wildlife professionals to risks, early detection and contingency measures during an outbreak, improve knowledge of sampling, surveillance and monitoring strategies, discuss methods of control and prevention, and review epidemiologic investigation steps. The following steps generally characterize an epidemiological outbreak investigation:

- a. Verify that an outbreak is occurring
- b. Confirm the diagnosis
- c. Establish case definition
- d. Descriptive epidemiology
- e. Hypothesis generation
- f. Analytic epidemiology
- g. Preliminary control/prevention measures
- h. Communicate findings
- i. Establish disease surveillance/monitoring.

While these steps are listed in a logical, sequential order, it is important to understand that they may actually take place simultaneously. A confirmation of the diagnosis may occur at the same time as the formation of the case definition. While the epidemiologic investigation is ongoing, it is quite likely that preliminary control and prevention measures will be underway. Communication with the work force, laboratories, government officials and other stakeholders and partners is critical throughout and after the outbreak. Some steps may require continuous action and adjustment (communication and control are typically ongoing, dynamic steps during an outbreak investigation).

a. Verify that an outbreak is occurring

Due to the large amount of resources that may be needed for a proper outbreak investigation, it is imperative to determine that there is an outbreak occurring. There are examples from state and federal entities where an epidemic was reported, but over time turned out to be a "false alarm: caused by change in laboratory personnel with false identification of an infectious agent, change in reporting thresholds, change in testing protocols, or reporting bias that led to over reporting of cases in a defined geographic region. Comparison with historical data is important in determining if an event is outside of what is normally expected. It may be necessary to respond and have "boots on the ground: to determine if an outbreak is truly occurring, but this step should not be skipped prior to deployment of large numbers of personnel, movement of any equipment, etc. It is also important to keep in mind that even in a confirmed wildlife mortality event; infectious disease may not be the only possible cause. Atoxic event (natural or intentional), trauma, severe weather, electrocution may also be causes of large scale mortality events, or the event may be multifactorial.

Wildlife

Blackbirds down

Jan 5th 2011, 16:28 BY THE ECONOMIST ONLINE



Incidents of mass wildlife mortality are quite common

THREE thousand blackbirds fell from the sky in the American state of Arkansas on New Year's Eve. The cause has not yet been determined but preliminary laboratory tests revealed acute physical trauma. Fireworks may have been to blame. While the incident was alarming, it is not unprecedented, according to America's National Wildlife Health Centre which monitors [wildlife mortality events](#) across America. One of the biggest such events occurred in 1986, when 30,000 Ohioan blackbirds and starlings were poisoned over a period of eight days. Our chart shows the incidents that caused the most wildlife fatalities in 2010.

b. Confirm the diagnosis

Cause of death determination will require laboratory confirmation. Many outbreaks are caused by previously known infectious agents that move into new hosts, new geographic ranges or have a strain mutation that causes increased virulence and can lead to death. In some cases, a wildlife disease outbreak may be due to a previously unknown agent, and research will be required for discovery, characterization and naming of the causative agent. As an example, white nose syndrome (WNS) caused by *Pseudogymnoascus destructans* is an emergent disease in the United States. While the initial agent and cause of death was not known, an outbreak investigation occurred to evaluate the extent of disease and species affected. After extensive diagnostic work the causative fungus was eventually isolated.

Early detection and rapid and accurate assessment of the causes of disease outbreaks are essential to effective disease control operations. Prompt submission of appropriate specimens to a qualified diagnostic laboratory is necessary for early problem identification, and prior communication with the laboratory is essential.

c. Establish Case definition

What details are needed to establish a case definition? (See OIE guidelines and established case definitions for known wildlife diseases.)

1. Population
2. Place
3. Time-frame
4. Geographic distribution/ limits
5. Clinical signs
6. Laboratory confirmation (confirmed, suspected, probable case)

Links to available sample case definitions from United States Department of Agriculture, Veterinary Services, Animal Plant Health Inspection Service:

- Eastern Equine Encephalitis:
https://www.aphis.usda.gov/animal_health/downloads/animal_diseases/eee-case-definition.pdf
- West Nile Virus:
https://www.aphis.usda.gov/animal_health/downloads/animal_diseases/wnv-case-definition.pdf
- Novel swine enteric coronavirus disease (SECD) including porcine epidemic diarrhea virus (PEDV):
https://www.aphis.usda.gov/animal_health/animal_dis_spec/swine/download/secd_case_definition.pdf

d. Descriptive Epidemiology

The outcome of descriptive epidemiology studies is to provide information on the frequency and distribution of disease. There is no pre-conceived hypothesis when performing descriptive studies. Descriptive studies seek to determine the extent of the outbreak:

1. Who (what population is affected?)
2. What (infectious cause or something else?)
3. Where (geographic extent of the problem)
4. When (time-frame of the event? Is it still occurring?)

Descriptive epidemiology studies are used at the beginning of an outbreak to try to determine initial risk factors to help researchers form early hypotheses. These are generally quick studies, taking up less time and resources than more complicated analytic studies. Descriptive studies do not generate risk or odds quantification; they do not establish causal factors. Many epidemiology text books cover the fundamentals of descriptive epidemiology, and therefore, we will not go into extensive detail on this topic. In brief overview:

1. Correlation (ecologic) studies: collect data at the population level, with the goal of showing correlation between two factors. This study will produce an R-score, ranging from -1 to +1.
2. Case reports: Report of a single case of a new/ interesting/ unreported disease or infection. This serves as the interface between diagnostic or clinical findings and epidemiology.
3. Case series: Reports pooled data from multiple cases of the same or similar diagnosis. Case series may help researchers form a hypothesis about causal and risk factors, but there is no statistical risk established by a case series.
4. Questionnaire survey: Questionnaire surveys can be used to determine frequency of disease with related factors at a point in time. These may be repeated to monitor changes in prevalence. Cross-sections of the population can be measured in their differences in exposure. Outcomes may reveal differences in age, exposure, seasonality, geography or species.

e. Hypothesis Generation

Following descriptive epidemiology (which is intended to be used at the beginning of an outbreak to identify further areas of research), the field team should have some risk factors that may be worthy of further investigation. Clinical and laboratory data may provide information if an infectious agent is responsible for the wildlife mortality event. Combining laboratory results with known pathology of the agent, incubation period information, any known reservoirs of the disease, potential sources of the infection, routes of transmission, and clinical picture/ classical presentation of the disease are important.

For example, following a descriptive epidemiological investigation of a rabies outbreak in Ethiopian wolves (*Canis simensis*), epidemiologists hypothesized that wolves in direct contact with domestic dogs (*Canis lupus familiaris*) are at higher risk of rabies compared to wolves lacking contact. This hypothesis regarding variability in contact rates can be tested using analytical epidemiological approaches described below.

f. Analytic Epidemiology

In contrast to descriptive studies, analytic epidemiology is more in-depth, and intended to validate or reject a working hypothesis. In some outbreaks, the descriptive epidemiology rapidly points to a particular source of infection, and further analysis may be unnecessary. However, in some wildlife events, or in events that may affect wildlife and domestic animals or humans, the source may be unclear and analytic epidemiology must be used to formally test the hypothesis.

Three general study designs can be used in analytic epidemiology:

1. Cross-sectional study-measures both exposure and outcome at the same time, and determines the relationship between groups. This measure is frequently used in wildlife investigations, but cannot be used to determine causal factors.
2. Case-control study: This type of study starts with identifying groups that are diseased (cases) vs. those without disease (control) and looks retrospectively to compare prior exposures. In wildlife scenarios, getting prior exposure

information is difficult, making case-control studies complicated and frequently inaccurate. Wildlife biologists may use study factors under current conditions as a proxy for the conditions at the time of the outbreak.

3. Cohort study: This type of study compares 2 cohort populations (exposed and unexposed) and follow those groups forward in time to compare the development (incidence) of disease.

In our example of a rabies outbreak in Ethiopian wolves, we could use a cohort study to investigate the role interactions with domestic dogs may have on the risk of rabies infection. This could entail tracking movements and rabies infection status of wolf pups through time, and estimating their usage of human occupied regions with large domestic dog populations. This would permit the estimation of the incidence of rabies infection relative to contact rates with domestic dogs.

g. Preliminary control/prevention measures

Control of diseases in wildlife is a challenge, and actions may not have the same impact or feasibility as one would expect in an outbreak of domestic animal disease. Control mechanisms may include: altering the path or likelihood of transmission by breaking the chain of infectivity. Going back to the basic epidemiology triad or vector-borne triad, for a control strategy, you must alter the host population, the agent, the environment or the vector.

Wildlife that have died of the disease are often a primary source of the disease agent, and for most situations their carcasses need to be removed from the environment to prevent disease transmission to other animals through contact with or consumption of the infected carcass. Carcass removal should be planned to avoid mechanical contamination of surrounding areas. Additionally, a plan for carcass disposal needs to be in place and logistically feasible prior to movement of any carcasses. The primary goal of carcass disposal is to prevent spread of the disease agent to other animals through environmental contamination. Incineration, burial, rendering and composting are 4 viable options for carcass disposal.

In addition to removing dead or diseased animals from the area, it may also be necessary to protect the live and apparently healthy animals during disease control activities. Depending on circumstances of the outbreak, consider denying animal use of specific sites by dispersing animals from the problem area, concentrating and holding wildlife within a specific area or trapping animals for sampling. Scare devices (propane exploders or cracker shells) may be useful for keeping wildlife away from a toxic or infectious event. Hazing with airplanes, helicopters, airboats, snowmobiles or other motorized equipment can successfully move wildlife away from disease problem areas. Consideration should be given to methods of concentration, such as attracting wildlife to an area with food or water. Animal relocation can be considered to remove animals from a hazardous region while carcass removal, disposal and disinfection efforts are ongoing.

The purpose of disinfection is to prevent the mechanical transmission of disease agents from one location to another by people, equipment and supplies. Some viruses, bacteria and other infectious agents have considerable environmental persistence. Diseases caused by prions (chronic wasting disease, scrapie, bovine spongiform encephalopathy) are among the most difficult to disinfect; ongoing research continues to investigate options for environmental decontamination in known prion-infected

areas. Disease control specialists should be consulted to determine the best options for disinfection of an outbreak site. Disinfection of the local environment involved in a disease outbreak may be required to prevent recurrence of the disease when the site is used by other animals.

Insect control may be an option in regions where vector-borne disease is of concern. In addition, and treatments or prevention that may affect the susceptible host (oral vaccine for rabies, for example) may be considered for high consequence diseases.

Human behavior may need to change as well to be successful in outbreak investigation and control. Limiting access to certain areas (e.g. quarantine), avoiding transporting agents accidentally from an infected to naïve area, and avoidance of humans (e.g., researchers) acting as mechanical vectors. An example of this is closure of caves in areas of concern to prevent the introduction and movement of the causative agent of white nose syndrome.

h. Communicate findings

Communication cannot be overstated in its importance. Communication to agricultural or public health officials should be initiated in the case of an agricultural or zoonotic disease, or if there is any risk to food safety. Environmental officials should be contacted in the event of a toxic chemical or other environmental contaminant. Information that is made available to the public should be prompt, accurate, and in non-scientific jargon.

Tips for successful communication to the public in the event of a large-scale mortality event (taken from the Centers for Disease Control and Prevention Crisis, Emergency and Risk Communication course material):

- Be first. Be right. Be credible.
- Build trust and credibility by expressing empathy, caring, competence, expertise, honesty, openness, commitment and dedication. "We too are upset to learn of the discovery of the antelope herd. It's a difficult sight to see. We're committed to finding out the cause of their death.:
- Don't over-reassure. It's important to give only the information available at this time.
- Explain the process in place to find answers: We have 3 teams of experts working to determine the cause of death in this region. We are working with university, police and international officials.

Due to variances in governmental structures, the responsibility of managing pathogens and disease in wild animals is often not clearly assigned. Certain pathogens may fall under the Ministry of Health, while others may fall under the Ministry of Agriculture and Veterinary Services. Responsibility for managing wild animal populations may frequently fall under the Ministries of Environment, Forestry or Fisheries. There may be confusion as to which branch or branches of government should be responsible for outbreak investigation, response and communications. Often, successful programs are achieved through inter-ministerial or inter-departmental collaborations which agree on objectives and define the roles and responsibility of each entity. In particular, pre-established governance structures and response plans to manage outbreaks can be helpful. During the hectic period of an

outbreak response, daily communication between any involved agencies should be expected. It is recommended that these roles and responsibilities are established well before an outbreak occurs, and that points of contact (POC) are known and easily located to facilitate communication.

i. Establish disease surveillance / monitoring

As the outbreak investigation winds down, you will need to determine your plans for continued disease surveillance and monitoring. As resources tend to be limited and careful thought placed on resource usage, consider the following questions:

- What work will continue into the next phase?
- How will you monitor for control of disease?
- How will you setup and design your surveillance plan?
- What additional tools and resources are needed?

III. DATA VISUALIZATION

1. Purpose

During outbreak investigations, it is often important to be able to display the geographic location of the outbreak(s). This information can be used to communicate with other jurisdictions, agencies and stakeholders to promote situational awareness, assess risk to valuable resources and public health, monitor spread, and assess management options. Data visualization can be as simple as placing a point on a map, or as complex as developing a Geographic Information Systems (GIS) project for the outbreak. The choice of data visualization platform depends largely on available resources, type, severity and associated risk of the outbreak, and overall objectives of the investigation. We will demonstrate simple data visualization techniques using readily available open-source software that can be used by anyone. Additionally, we will provide resources and information for the more detailed GIS applications; however, covering these applications in detail is beyond the scope of this training.

2. Google Earth

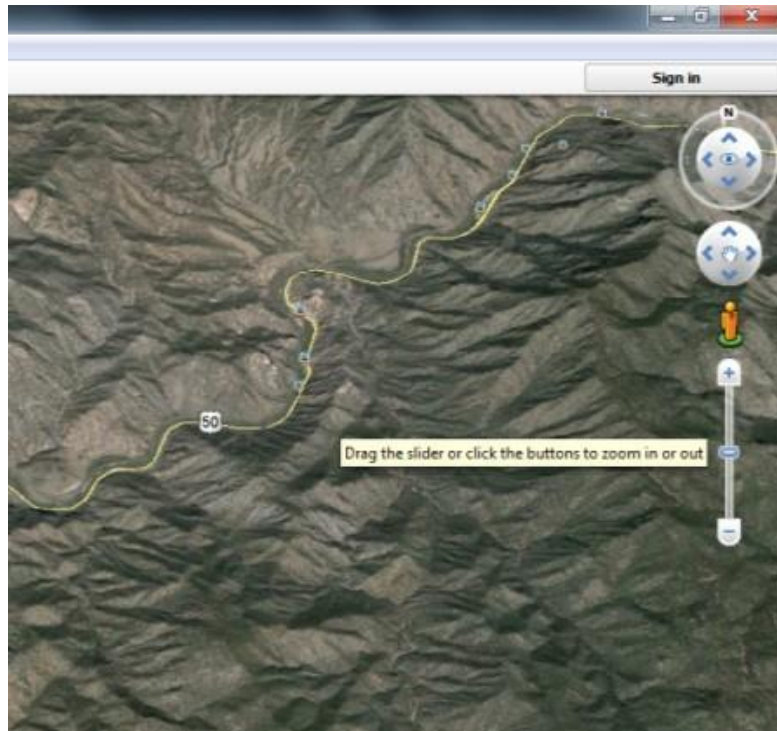
We will begin by demonstrating how to use Google Earth Pro to create shareable maps of known outbreak locations. Google Earth Pro is a freely available software package that is used for a wide variety of applications, and it can be downloaded at the following: <http://www.google.com/earth/download/gep/agree.html>. An e-mail address and the following key GEPFREE are needed to use this software after installation.

3. Single Point

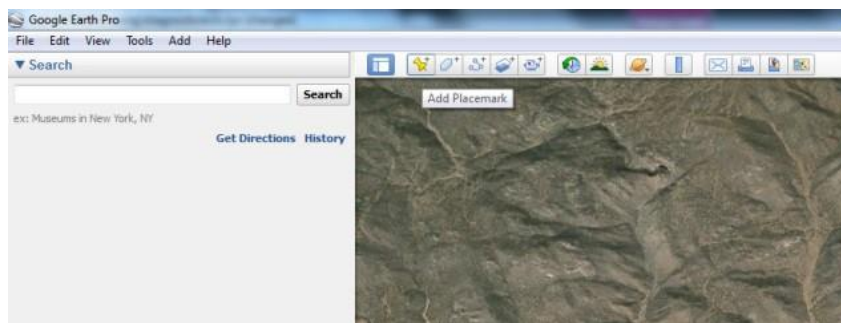
We will begin by demonstrating how to place a single point (i.e., a single outbreak location) on the map using the following step by step instructions:

1. Open GoogleEarthPro by clicking on the icon.

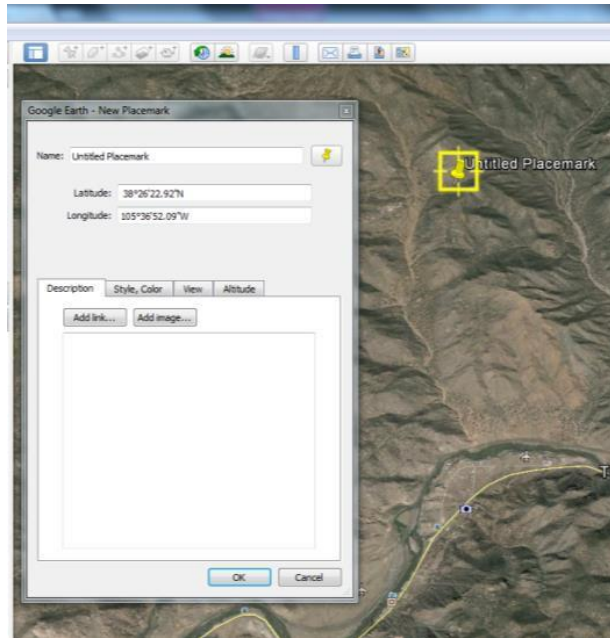
2. Use the mouse to navigate to the desired location on the globe. You can zoom into the desired location using the slider on the top right hand side of the screen.



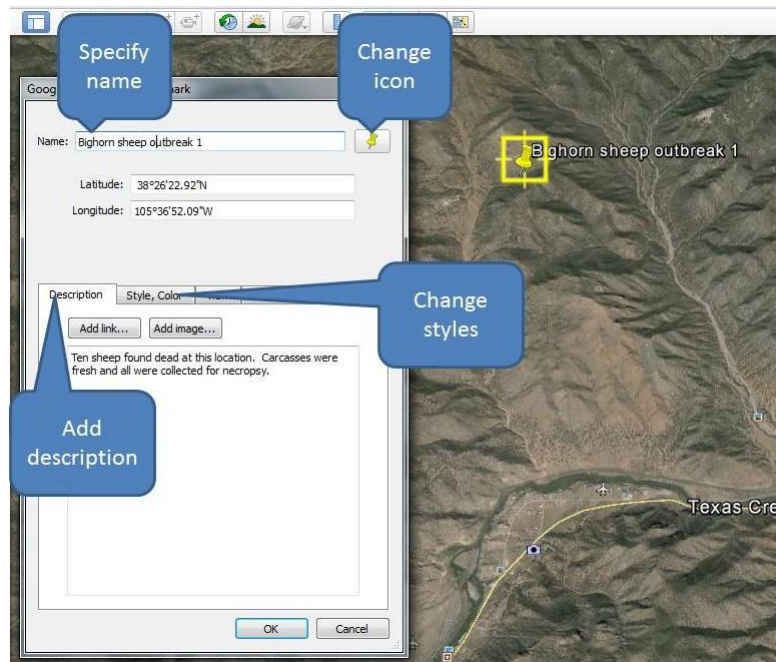
3. Once you have navigated to the location of the outbreak it is time to place a marker at that location. Go to the top of the "map window" and click on the yellow push pin or add place mark button.



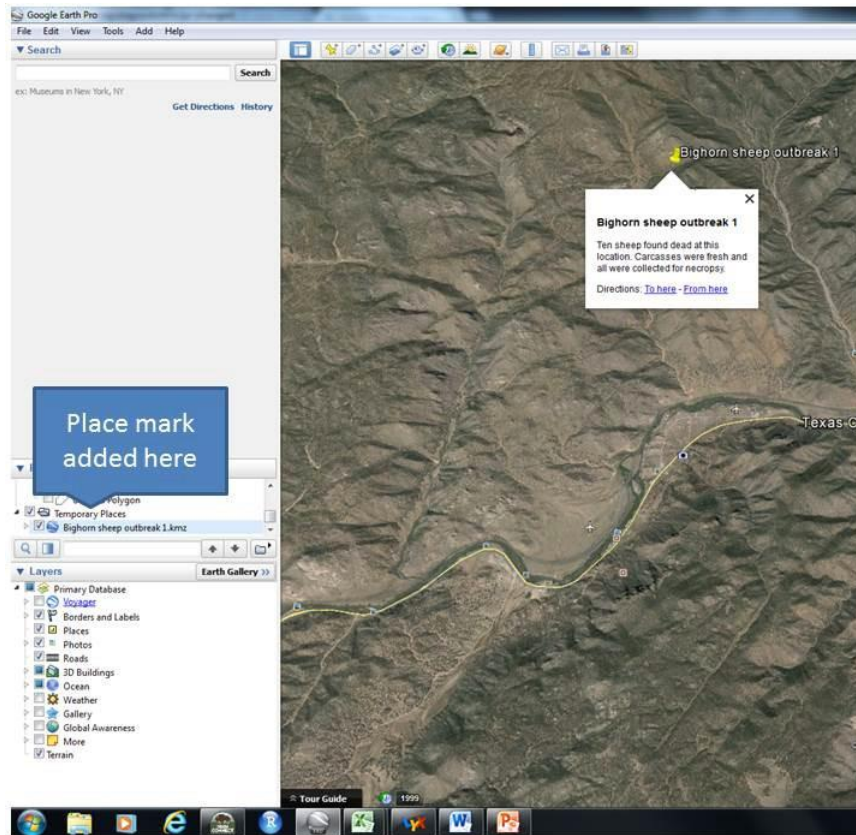
4. This will create a blinking yellow box around a new place marker. This new place marker can be moved to a precise location by clicking and holding down the left mouse button, and then dragging the place marker to the desired location. Once it is in the location release the left mouse button. Additionally, a dialog box pops up that allows you to link the place marker to useful information.



5. You can pick the icon you are using for your place marker by clicking on the "push pin: icon in the upper right corner of the dialog box. You can name your place marker by clicking in the text box following "Name: within the dialog box, and typing an informative name (e.g., Bighorn sheep outbreak 1). If you would like to add additional descriptive information, go down to the "Description: tab in the dialog box. Click in the text box within this tab, and add in your desired information. You can change the style and color of your icon and its associated label by clicking on the "Style, Color: tab in the dialog box. Lastly, there are other options dealing with appearance and mapping in the additional tabs of the dialog box.



6. When you are finished click the "Ok" button at the bottom of the dialog box.
7. Now you can click on the place marker and the associated description you entered is displayed. If you would like to edit content of the description, change the name or style, etc., simply, right click on the icon for the place marker. This will open the dialog box previously described, and edits can be made as necessary.
8. Now supposed you would like to share this file with colleagues or stake-holders. Save the file by going the File Menu in the top left of the screen. Scroll down to "Save:" and click. There are several different options available. You can save the file as an image, or you can "Save Place As:" in a shareable file format. Let's choose the latter by clicking on "Save Place As:" option. We enter a name for our file in the pop-up dialog box. Files can be saved in either .kmz format (compressed file similar to winzip and will include any image overlays, etc.) or a .kml (Google Earth file format, uncompressed). We will save the file in the as outbreak1.kmz.
9. This file can now be sent to anyone with access to Google Earth. They can open the object by going to the File Menu in the top left of the screen, and choosing Open. This will allow them to "browse:" to the location on their computer of the desired file. They can click on the file, and it will add it to their "Places:" on the left-hand side of the screen.



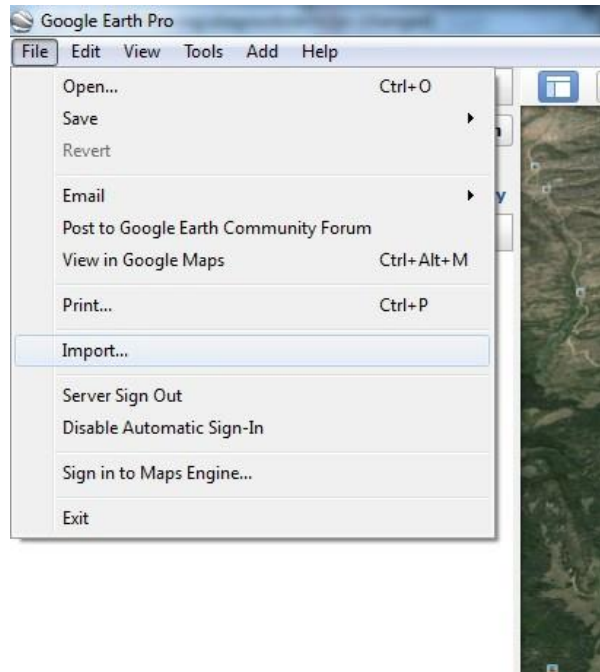
10. And that is how it is done!

Create From Table

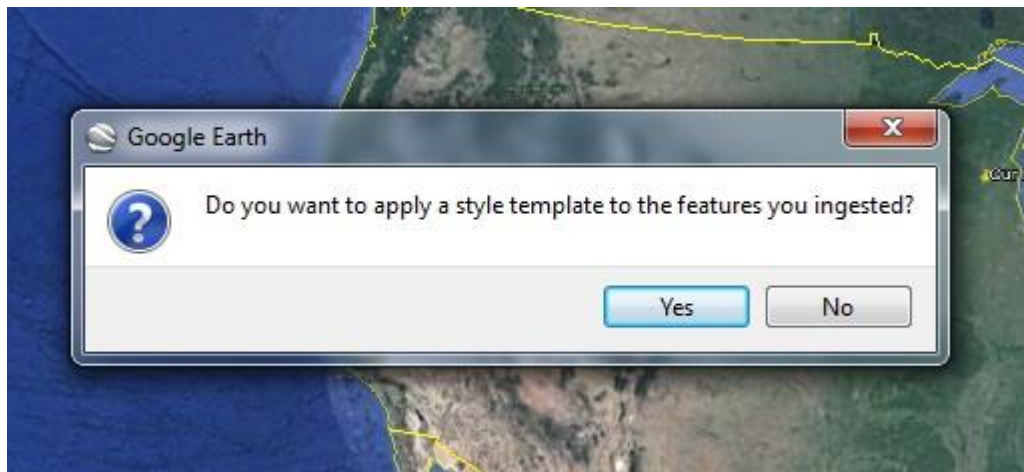
We have now introduced you to the basics of using Google Earth Pro to share location information for a single outbreak. However, often times you may have multiple outbreaks at multiple locations or the individual locations of moribund, or dead animals in a given outbreak have been recorded. Following the above procedure for each location can become tedious as the number of locations increases. In this section, we will demonstrate how to create place markers in Google Earth Pro from a table.

1. Create a table of the locations of interest if needed. Google Earth Pro uses the geographic coordinate system (i.e., latitude and longitude) on the World Geodetic System of 1984 (WGS84). So locations should be specified in this format; however, Google Earth will recognize imported coordinates that are in degrees, minutes, seconds (DMS), decimal degrees (DDD) or degrees, minutes, with decimal seconds (DMM). Below is the table of data points we will use for illustration specified in DDD.
2. Once you have created/received your table. Open it in a spreadsheet software, and save it in comma separated values (.csv) format.
3. Now open Google Earth Pro and click on the File menu in the top left corner. Scroll down and click on "Import:."

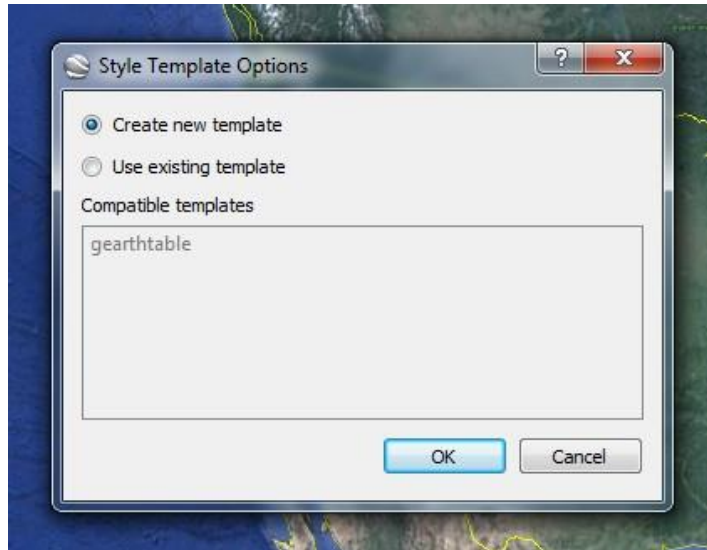
Species	Sex	Age	Date	Number dead	Latitude	Longitude	Comments
Bighorn sheep	M	yearling ram	6/25/2016	1	37.600398	-105.102344	citizen reported
Bighorn sheep	F	2 lambs	6/25/2016	2	37.611498	-105.085276	one lamb moribund, other dead
Bighorn sheep	F	7+	6/31/2016	1	37.587884	-105.080647	found by investigating biologist
Bighorn sheep	F	all 4+	6/31/2016	4	37.648576	-105.089739	found by investigating biologist
Bighorn sheep	F	lamb	6/31/2016	1	37.623723	-105.047152	found by investigating biologist
Bighorn sheep	F	4+,yearling	6/31/2016	2	37.601617	-105.163026	found by investigating biologist
Bighorn sheep	F	yearling	6/31/2016	1	37.633389	-105.068236	found by investigating biologist
Bighorn sheep	M	4	7/1/2016	2	37.59999	-105.07778	found by investigating biologist
Bighorn sheep	F	lamb, 3	7/1/2016	2	37.61231	-105.15489	found by investigating biologist



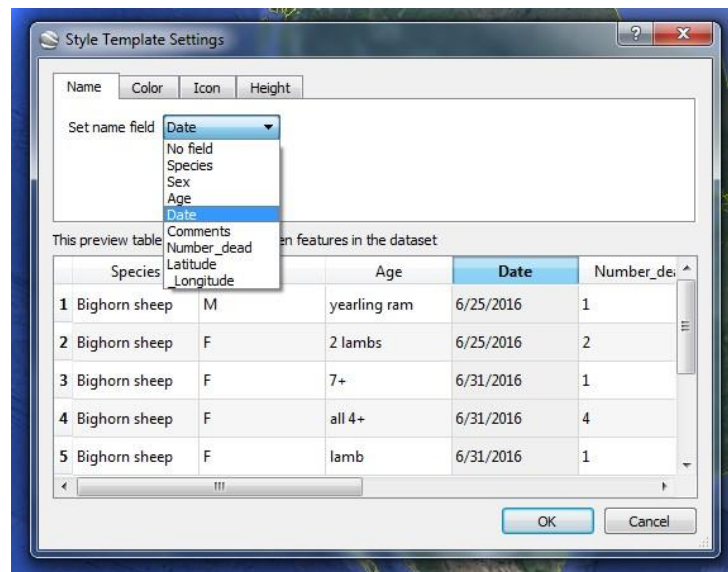
4. This will cause a dialog box to open where you can browse to the .csv file you created in Step 2. Select the desired file and click "Open:.
5. The following dialog box will open.



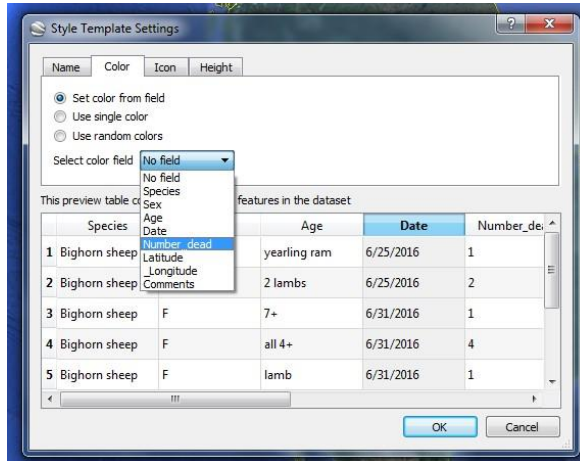
6. Select "Yes: if you would like to customize the display style and identifiers of the place markers for each location. If you select "No:, the default style will be used for the place markers and "no name: will be used for the identifiers. This is generally undesirable so we will select "Yes:.
7. A new dialog box will then pop up.



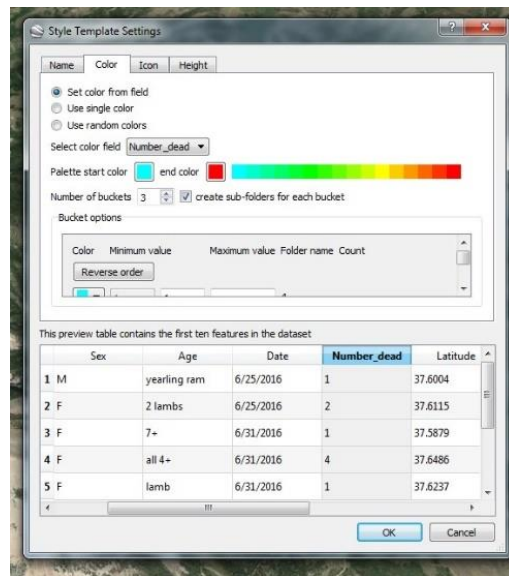
8. If you have an existing style template, you can select it here. However, we will create a new template by selecting "Create New Template"; and clicking "Ok".
9. The resulting dialog box contains a number of options for the style of place markers.
 - (a) First you can select a name that will be displayed for each place marker from among the field names in your imported table. In this example, I select the date field, which will result in the date being displayed above each place marker.



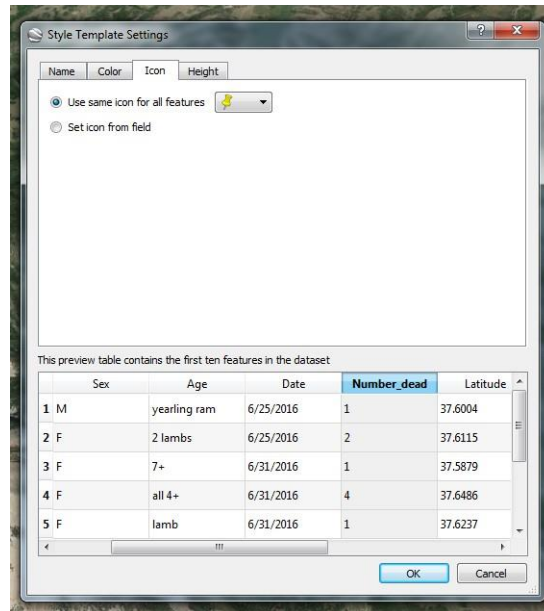
- (b) Next we will select the color of our place markers by clicking on the "Color:" tab in the dialog box. We have several choices. We can choose all the place markers to be one color, we can assign a random color to each place marker, or we can assign a color based on a field within our table. We will do the latter and use the "Number dead:" field by selecting the "Set color from field:" option. This will provide an easy display of the relative number of individuals discovered at each location.



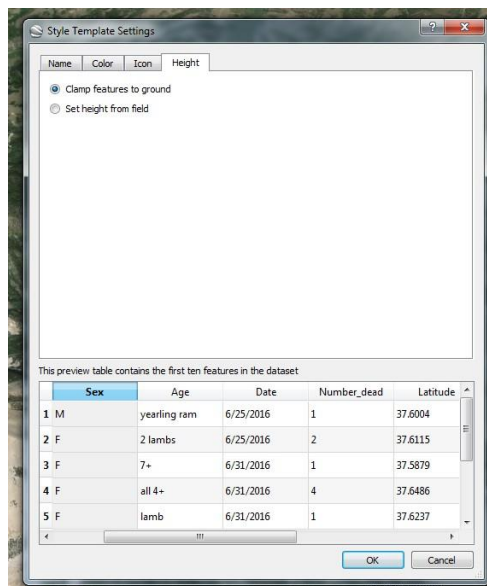
- i. Once we have selected the "Number dead: field. We can create our color ramp (i.e., the range of colors for differing values of "Number dead:). I will choose a light green as my "start color: and red as my "end color:
- ii. Next we have the option to choose the number of "buckets:. This is the number of groups you want to create based on the values in the "Number dead: field for example. We only have 3 different values so I want a unique bucket for each value; however, if we had 100 unique values we may want to only have 10 buckets and so Google Earth Pro would put values from 1 to 10 in the first bucket; values 11-20 in the second bucket, and so forth. You can also specify what numbers you would like in each bucket or reverse the order of the colors in the "Bucket options: section. For this example, we leave the number of buckets set at the default of 3. At this point, you also have the option to have a sub-folder created in your places drop down on the left-side of the screen for each bucket. This makes it easy to select groups of locations based on the "Number dead: for our example. So we will check that box.



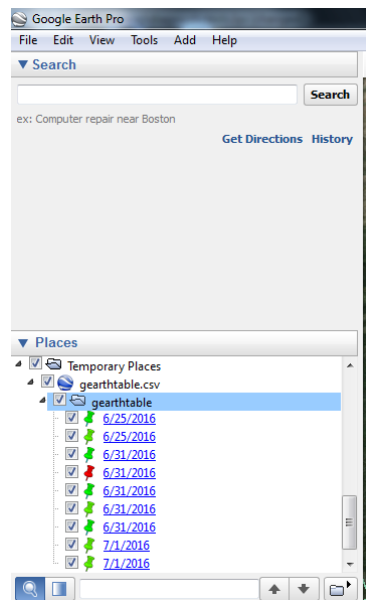
- (c) We now can select an icon style for our place markers by clicking on the Icon tab. Once again you can use an identical icon for each place marker or you can have the icon depend on a field in the table. We will use one icon for all place markers in this example. We will use a push pin icon.



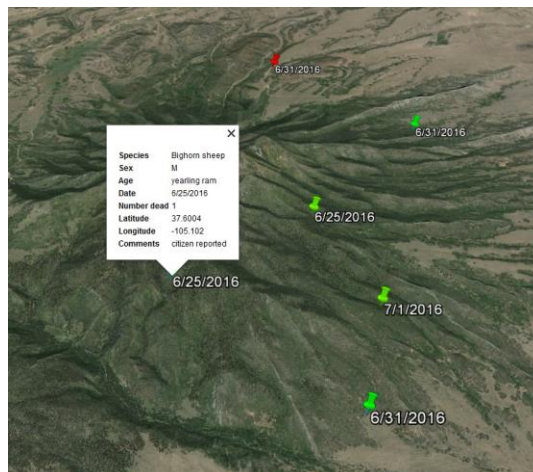
- (d) Lastly, we can choose how Google Earth Pro displays the place markers in relation to the height from the ground. We can specify the height based on a field in the table, or we can have each place marker "stuck" or "clamped" to its location in the ground. We choose the latter and so check "Clamp features to ground" option. This completes our style specification. So we finish by clicking "Ok".



10. We will save the new template file as prompted.
11. Our place markers have now been created. If they are not displayed automatically. Go to the Place drop down menu on the left of the screen. The newly created place markers will be listed under "Temporary Places:.". There will be a folder with the name of your .csv file. To turn it on click the box next to the name. This will turn on all your place markers. You can turn on/off individual place markers by clicking on the arrow next to the check box by the name. This will then display each place marker with its own check box. Clicking on the check box will turn it on/off.

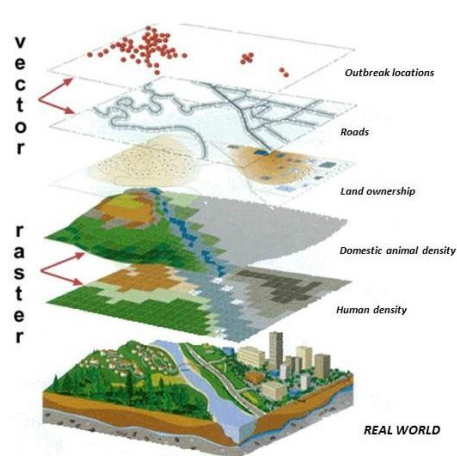


12. You can now scroll around view your place markers and you can retrieve the information associated with each place marker by simply clicking on each place marker.
13. You can save and share this file in exactly the same way as described previously for a single place marker.



We have presented a simple means to visualize and share outbreak data. However, often there is a need to do more complex data visualization by over- laying detailed and varying sources of geographic information. In these circumstances, Geographic Information Systems (GIS) are the most appropriate tool.

A GIS is a system designed to capture, store, manipulate, analyze, manage, and present all types of spatial or geographical data¹ for the purpose of understanding patterns and relationships. There is an ever- increasing amount of georeferenced data becoming readily available for use in outbreak investigations, and it has utility for assessing risk, extent of impacts and possible management interventions. A GIS provides the means to harness this wealth of information in an efficient and rigorous way. There are a number of software platforms that provide the tools and framework for creating a GIS. ESRI www.esri.com is a leading company that produces many GIS products that are used globally, and provide a wide range of functionality; however, licensing expenses can be high. There are also open-



source software for GIS construction. QGIS www.qgis.org/ is one example of these types of software that is widely used and provides much of the capabilities of the more expensive ESRI products; however, there are many open-source GIS applications with some excelling for specific applications. An Internet search is a good place to begin to evaluate the suite of potential tools available, and obtain instructional materials. We will not discuss GIS further in this workshop, but wanted to provide a brief introduction because of its potential to be an important tool in outbreak investigations.

4. R Project

We will continue our discussion on data visualization by demonstrating some of the features of the statistical program R for visualizing data. R is becoming the *de facto* computing program for scientific data analysis. It is open-source, and provides a wealth of geostatistical capabilities, which can be used to rigorously model features of the disease and outbreak processes. Our focus will be to demonstrate how to bring and display geospatial, outbreak data into R where sophisticated statistical analyses may be conducted (although such analyses are beyond the scope of this workshop). The R program and any necessary libraries (i.e., modules that can be loaded into R for particular analyses) can be freely downloaded from the CRAN website <https://cran.r-project.org/>. Our motivating example will be based on White Nose Syndrome (WNS) mortality events extracted from the United States Geological Survey's National Wildlife Health Center's (NWHC) event reporting tool, WHISPers.² This tool will be discussed shortly; however, we generated a .csv file from this tool containing spatial location and associated attribute data for each WNS event reported to NWHC since 2007. We conducted some post-processing, imported it into R, and created an R dataframe called "wnsout". We display an excerpt from the final table below.

¹ https://en.wikipedia.org/wiki/Geographic_information_system

² <https://whispers.usgs.gov/home>

```

## States Counties Event.ID Start.Date End.Date
## 1 AL Jackson 16432 2012-03-01 2012-04-15
## 2 AL Limestone 17169 2015-02-04 2015-04-15
## 3 AR Franklin 17133 2015-02-10 2015-04-01
## 4 AR Independence 17135 2015-01-16 2015-04-01
## 5 AR Marion 16840 2014-01-12 2014-04-01
## 6 AR Newton 17134 2015-01-31 2015-04-01
## Affected Species
## 1 71 Eastern Pipistrelle, Northern Long-Eared Bat
## 2 3 Eastern Pipistrelle, Indiana Bat
## 3 3 Eastern Pipistrelle, Big Brown Bat, Indiana Bat
## 4 2 Eastern Pipistrelle
## 5 5 Northern Long-Eared Bat
## 6 3 Eastern Pipistrelle
## Event.Diagnosis XCOORD YCOORD year
## 1 White-nose Syndrome -85.99936 34.77945 2012
## 2 White-nose Syndrome -86.98140 34.81010 2015
## 3 White-nose Syndrome -93.89064 35.51232 2015
## 4 White-nose Syndrome -91.56971 35.74157 2015
## 5 White-nose Syndrome -92.68422 36.26839 2014
## 6 White-nose Syndrome -93.21787 35.91997 2015

```

We will use the `ggmap` library to visualize the WNS event data in R. We will begin by plotting all the events in a "bubble plot": to depict the location and relative size of each event. We will use the following R code to create the plot. We will not go over the code in detail, but each line is annotated immediately after or below it describing its purpose and to permit understanding and reproducibility.

```

library(ggmap) #load necessary libraries
library(knitr)
#wnsout<-read.csv(wnsdata)
#command to import data from .csv file
#####Note it is currently commented out using 11#11

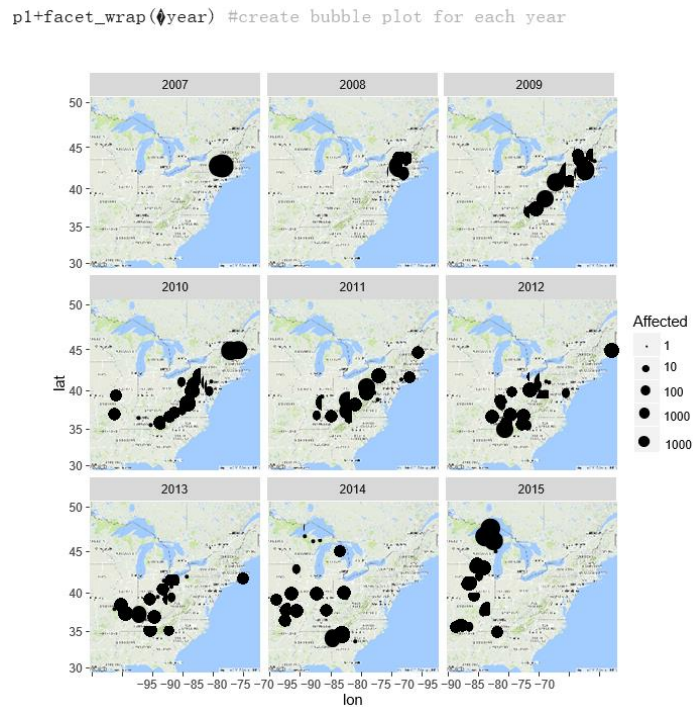
sbbox <- make_bbox(lon = wnsout$XCOORD, lat = wnsout$YCOORD, f = .01)
#create bounding box for data
map<-get_map(location=sbbox, maptype = "terrain",
             source= google ,color= color )
#pull the area interest from google maps

p1<-ggmap(map)+ #use map as background

geom_point(data=wnsout, aes(x=XCOORD, y=YCOORD, size=Affected, shape=21))+
#bubble plot of event locations/bubble sizes= based on # affected
scale_size_continuous(breaks=c(1, 10, 100, 1000, 10000),
                      trans="log10", range=c(1, 8))+
#create breaks in # affected for bubble plot sizes
scale_shape_identity()
#used to allow shape =21/open circles be used for shape
p1 #plot map

```

Examining this plot, it is clear that this may not be the best way to visualize the data, given the number of events that have occurred and the large degree of spatial overlap. So, we plot the information again, but now create a bubble plot for each year.



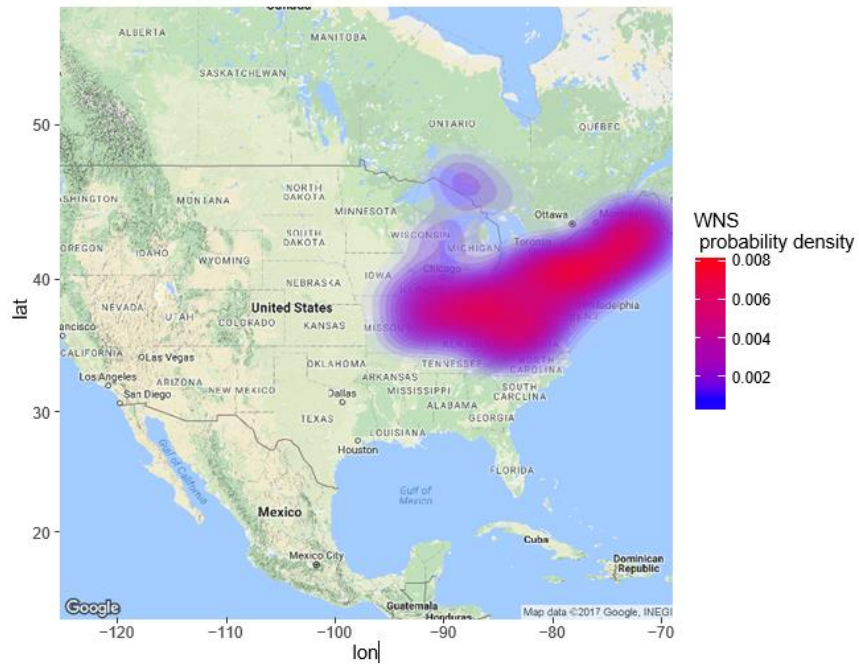
Alternatively, it may be desirable to have a map depicting the intensity of WNS events rather than the bubble plot representation. This is where the true power of R begins to shine. One simple way to create such a map is to use a kernel density estimation routine to create and visualize the desired intensity surface. This surface can then be overlaid on a map to provide the requisite geographical context. This is done by simply using the following few lines of code:

```
library(ggmap) #load necessary libraries
library(knitr)
#wnsout<-read.csv(wnsdata)
#command to import data from .csv file
# Note it is currently commented out using ll#ll

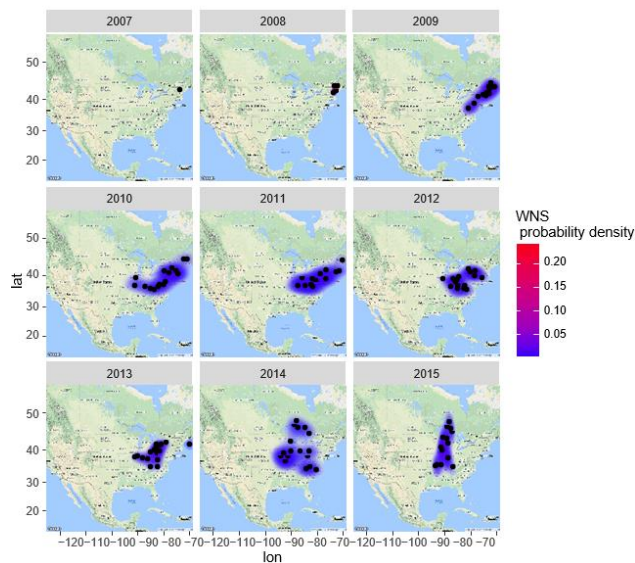
map<-get_map(location="eastern united states",zoom=4, maptype = "terrain",
             source= google ,color= color )
#pull the area interest from google maps

p2<-ggmap(map)+ #use map as background
  stat_density_2d(aes(x=XCOORD, y=YCOORD, fill=..level..), alpha=0.25,
                 data=wnsout, bins=20, geom="polygon")+
  #calculate kernel density of WNS events
  scale_alpha_continuous(range=c(0.1, 0.5))+
  #set transparency
  scale_fill_continuous(low = "blue", high = "red",
                       name="WNS\n probability density")
#set color ramp and legend title
p2 #plot map
```

We can also create an intensity surface for each year as we did for the bubble plots. This is facilitated by simply adding one additional line of code.



R has much more functionality for data visualization including being able to import .shp files (ESRI's GIS file format), and it has a wealth of statistical capabilities. Our purpose here is to provide a quick introduction to R and demonstrate a few of its capabilities for data visualization. The one down-side to R is the need to learn the programming language; however, there are many resources in print and on the Internet for using R if further instruction is desired.



USGS WHISPers: We will conclude by providing a brief description of a data visualization and situational awareness tool developed specifically for wildlife health data. The Wildlife Health Information Sharing Partnership - event reporting system (WHISPers) developed at the NWHC is a web-based repository for sharing and visualizing basic information about historic and on-going mortality and morbidity events in wild animals.



The system provides natural resource managers with timely, accurate information on these events to facilitate disease management and planning efforts. It can be accessed at <https://whispers.usgs.gov/home>. An example of the WHISPers interface is shown below.

Unlike databases in many veterinary laboratories, the structure of the WHISPers database was built around the concept of wildlife events rather than individual animals. Although determining the cause of an event does include diagnostic findings from a representative number of affected animals those findings are used in conjunction with epidemiological information (affected species, age and sex distribution, location, time of year, etc) to determine the suspected cause of the larger event. The primary driver for this concept is that wildlife disease management is generally enacted to protect populations rather than individual animals. For example, one individual in a colonial water bird colony may have an acute bacterial infection, but if 100s of birds at this site are dying of avian botulism, efforts would generally be targeted at the mitigating circumstances causing the outbreak of botulism.

The database currently contains over 40 years of records gathered by NWHC on wildlife mortality events, including event information shared with NWHC by state and federal partners. Although the system is one of the largest wildlife disease databases available in the U.S, information is opportunistically collected and voluntarily reported to NWHC. Therefore, it contains a subset of all the mortality events that have been documented in North America.

The image shows a detailed view of an event in the WHISPers system. The event is titled 'Event #17359' and is categorized as 'Mortality/Morbidity'. The status is 'Complete' and the dates are '9/28/2015 - 12/1/2015'. The number of affected animals is 900. The diagnosis is 'Trematodiasis (Leyogonimus polyoon) [major], Trematodiasis (Cyathocotyle bushiensis) [major], Trematodiasis (Sphaeridiotrema sp.) [major]'. The location is 'La Crosse Co, Wisconsin, United States'. A map shows the location of the event in Wisconsin. Below the map is a table of species affected by the event.

American Code	Common Name	Scientific Name	Population	Sick	Dead	Est. Sick	Est. Dead
		<i>Falco americana</i>			97		900
		<i>Apfya affinis</i>		1			

American Code	Common Name	Scientific Name	Population	Sick	Dead	Est. Sick	Est. Dead
		<i>Bucephala albeola</i>		1			900

The records in WHISPers can be searched by species, disease, location (to US county level), number affected, and event date. You can visualize the events on an interactive map as well as download the data into a .csv file. By selecting an event from your search you will be taken to a screen with additional information on the event including the agency level contacts and number affected for each species. A Frequently Asked Questions section and instructions for using and searching WHISPers are also available on the website.

Work is on-going to create a mechanism for additional partners to input information on wildlife mortality events thereby improving the temporal, species, and geographic coverage of this long-term dataset. It is important to note, the system is not intended for citizen science data collection, but rather verified information collected by biologists and diagnoses confirmed in a laboratory. The website therefore requires users to register as a contributor before they can enter data. Similar to creating "friend circles" on social media, users can create "user groups" where they can grant others access to view and edit their data. Data entry pages will include all of the diagnoses, species and location data fields as well as a field that determines if the event can be viewed by the public. Once these features are deployed, work will begin on incorporating analytical tools into the site.

IV. Personal Protective Equipment (PPE) and Biosafety During Wildlife Disease Field Investigation

1. Personal Protective Equipment

If you face the possibility of responding to a wildlife mortality event, it is imperative to prepare ahead of time to decrease the risk of disease transmission to personnel, and to ensure proper sample collection, handling, and submission. It is equally as important to have the appropriate equipment, as it is to know how to properly don and doff personal protective equipment (PPE). As a general rule of thumb, if there is a possibility that the wildlife mortality event was caused by an infectious agent, it is the responsibility of the investigation team to wear PPE and to assist others in the field with proper protection. Availability of material may vary from region to region. For the purposes of this training, we will not focus on any specific brand of PPE; but rather the fundamental purpose of protection. It is worth noting that the time to obtain PPE and to train on proper donning and doffing procedure is well ahead of an actual mortality event. A knowledgeable and trained staff should also know about the types and location of PPE for his/her agency in order to safely respond in a much more rapid fashion.

Note: The type of PPE will vary based on the level of protection required.

a. Options for Personal Protective Equipment in field studies

Commonly used materials include: masks, gloves, boots, coveralls, respirators, and goggles. This equipment puts a barrier between you and the disease agent. The level of protection required depends on the situation.

Suggested equipment: Ready to go in a field investigation box

- Rubber boots or other washable/disinfectable shoe covers
- Gloves- multiple sizes, latex and nitrile
- Gowns
- Respirator or face mask
- Face shield or eye protection
- Disinfectant and application instruments (sprayers)

Methods for PPE use should be weighed against the known or suspected cause of disease, zoonotic risk to workers, possible routes of transmission, and any available prophylaxis or treatment. PPE also needs to be weighed against practicality, and burden to the worker in cases of extreme weather. In very high temperatures, workers should not be expected to don full coveralls, respirators, and face shields for any excess amount of time, due to the concern of dehydration or heat stress.



Before you begin working with or around wildlife, ask yourself:

- What are the potential risks or hazards in my areas and/or with the species I will be working with? What are the clinical signs of these diseases in humans and wildlife?
- Because of the risks, what PPE will I need before beginning work? Do I have the necessary skills and resources? Outline a plan in a job hazard analysis and discuss with a supervisor.
- Do specific health concerns exist for me or any members of my team? Conditions may include pregnancy, a compromised immune system, etc. Is there a need for pre-exposure vaccination? Discuss specific health concerns with your physician.
- Do any red flags exist? (See below)

Red Flags:

Multiple dead animals of unknown cause, blood coming from any orifice (nose, mouth, rectum, etc.) without any signs of trauma, presence of pus-filled lesions in the lungs, or any animals exhibiting neurologic signs.

Standard safety precautions while working with wildlife or handling potentially infectious materials:

1. Always use protective barriers- this may include gloves.
2. Wash hands thoroughly with soap and water after removing gloves.
3. Disinfect soiled equipment and contaminated surfaces or items.
4. Do not eat, drink, smoke, or chew gum while handling wildlife.
5. Work in well ventilated areas when indoors, and upwind of specimens while outdoors.
6. Avoid needle sticks and cuts. Prepare for and use safe containers to dispose of "sharps"

7. Transport and store samples properly and safely. Label biologic samples appropriately.
8. Seek medical attention when ill and inform provider of potential disease exposures.

Pathogens from wildlife are transmitted by three major routes of exposure: contact, aerosol, and vectors. Consider each of these routes for your planned activity, and how you can protect yourself in each scenario.

b. Contact

The single most common source of transmission is through contact, particularly touching your eyes, nose or mouth with contaminated hands. The most effective tool for protection is good hand hygiene:

- Wear gloves when making contact with animals, their fluids, or a contaminated environment.
- Always wash hands with soap and water (for at least 20 seconds) after removing gloves. This is especially important before eating, drinking, smoking or using a cell phone.
- Hand sanitizer may be useful in the field when you are unable to wash hands immediately.
- Coveralls should be considered when clothing or materials may be contaminated.
- If your work may generate splashes or droplets, protective goggles or a splash guard should be part of your plan to decrease contact.

c. Aerosol

We recommend always working in a well-ventilated area and working upwind of a potentially contaminated area or carcass. In addition, in circumstances where the species or geographic range may include high-risk pathogens and activities that are likely to aerosolize pathogens, additional PPE may be necessary.



Situations requiring respiratory protection include:

- Conducting a necropsy on a suspected plague animal.
- Field collection or necropsy of any carcass when aerosolization would present danger.

Wearing a respirator may require medical clearance, and should be fitted to ensure a proper seal. Standard dust masks look similar to respirators, but do not filter the same amount and may not protect you against infectious agents.

d. Vector

Vector-borne diseases vary by geographic region, and the types of vectors, pathogens and hosts that may be present. Strategies to help protect you from vector-borne disease:

- Wear an insect repellent containing DEET and/or wear permethrin treated clothing.
- Wear long pants and sleeves, and tuck pants into socks. Or wear coveralls with elastic wrist and ankle closures.
- Conduct tick checks immediately following field work.
- Shower within two hours of returning from the field. Clothes should be placed in a dryer if possible to kill any ticks.

e. Pre-exposure vaccines

In regions where rabies virus is present in the mammalian populations, pre-exposure rabies vaccine should be considered for wildlife personnel. Pre-exposure vaccination does not eliminate the need for medical evaluation after a potential rabies exposure; however, it does simplify and reduce the costs and amount of post-exposure treatment. Personnel at risk of rabies exposure may include persons that handle bats, those who may be responsible for investigation of any mortality event involving terrestrial rabies, or anyone responding to an event with undiagnosed neurological signs. In the case of exposure to rabies virus, once an individual is showing signs of infection, this leads to a universally fatal disease progression.

In addition to vaccination for rabies, it may be appropriate to consider vaccination for other diseases (for example: small pox vaccine is protective against monkeypox) and to have an occupational health plan with availability for antibiotics and antiviral medications.

At the USGS National Wildlife Health Center, we have our biologists and biology technicians carry a medical card with information regarding possible exposures. If appropriate, as part of the occupations safety plan in your entity, consider creating a similar type of card to assist physician to detect zoonotic diseases in field workers who fall ill. See example of card here:

ATTN: Medical Personnel

This person works with wildlife and may have been exposed to certain zoonotic diseases not routinely considered in the differential diagnoses of febrile illnesses. In case of sickness in this individual, please consider zoonotic diseases including, but not limited to the following:

Anthrax, Arbovirus encephalitis, Brucellosis, Giardiasis, Hantavirus, Hendra Virus, Highly Pathogenic Avian Influenza, Histoplasmosis, Leptospirosis, Lyme Disease, Monkeypox, Mycotoxicosis, Nipah Virus, Psittacosis, Q Fever, Rabies, Rocky Mountain Spotted Fever, Salmonellosis, Sylvatic Plague, Tularemia, Typhus, & West Nile Virus.

(continued on back)

For more information on the occurrence of these diseases in humans, please contact:

The Centers for Disease Control and Prevention
1600 Clifton Rd.
Atlanta, GA 30333
1-800-232-4636 (1-800-CDC-INFO)
<http://www.cdc.gov/>



For more information on the occurrence of these diseases in wildlife, please contact:

USGS National Wildlife Health Center
6006 Schroeder Rd.
Madison, WI 53711
(608)-270-2400
<http://www.nwhc.usgs.gov/>



An excellent resource for safe practices in wildlife work may be found at the following website: https://www.nps.gov/subjects/policy/upload/RM-50B_Chp54_Safe_Work_Practices_Handling_Wildlife-508.pdf. Used with permission by the United State Department of Interior National Park Service.

2. Biosecurity

During the Annual World Assembly of Delegates at the 83rd General Session of the OIE (24-29 May 2015), several new terms were incorporated into the Terrestrial Code, including the definition of biosecurity. Definition of biosecurity: The implementation of measure that reduce the risk of the introduction and spread of disease agents.

Proper cleaning and disinfection of equipment and vehicles may vary depending on the causative agent of the outbreak. Options for cleaning are detergent, soapy water, steam, spray disinfectants, UV light (sunlight), and desiccation.



Contamination from some agents are very difficult to nearly impossible to correct (prion diseases in particular) and should be considered prior to taking equipment and instruments into the area. When planning a response to a wildlife outbreak, it is imperative that the field investigation team does not carry the agent from an infected area to an uninfected area. Traffic and researchers should, if possible, mobilize separate teams with separate vehicles, equipment, and sampling materials to avoid any risk of cross-contamination.

3. Biosafety

The application of knowledge, techniques, and equipment to prevent personal, laboratory, and environmental exposure to potentially infectious agents or biohazards. Personnel leaving a suspected outbreak area should discard PPE, or decontaminate, double-bag, and take PPE offsite. Any equipment that may be taken into an infected area should be considered with caution. Safety measures should be taken to protect equipment that may not be easy to decontaminate (i.e., digital camera, GPS device, communication radios, or phones). One option is to seal these items in clear plastic bags that can be disinfected along with PPE. In addition to PPE, all potentially contaminated materials should be discarded safely if possible, or properly disinfected if non-disposable. Instruments, equipment, and vehicles should be considered when developing a biosafety plan during an outbreak response.

4. Biosecurity point

A designated area for putting on and removing PPE (also known as donning and doffing personal protective equipment) at the border between clean and contaminated areas. This point may include an area to wash hands and boots. A footbath or boot-washer facility should be available. This point should include disposal bags for contaminated PPE. The biosecurity point is an imaginary boundary between the clean and dirty sides, which should be demarcated. There may also be an area of limited traffic onto the suspect area, if the geographic extent of the outbreak has been established.



V. Diagnostic Tests

1. Purpose

Previous discussions have centered around outbreak investigations and the logistical considerations associated with the outbreak. We will now consider diagnostic tests in relation to outbreak investigations. It is worth noting that many of the considerations discussed herein are also relevant for diagnostic test selection for use in surveillance activities; however, we will frame our discussion in terms of outbreak investigations. It is critical before performing diagnostic tests to clearly establish the requirements for conducting a particular test. Although the purpose in an outbreak investigation is to establish the causative mechanism underlying the outbreak, nevertheless some key questions that should be answered prior to initiating diagnostic testing include:

1. What auxiliary information is available to assist in test selection?
2. Has the test been validated in the host species and pathogen of interest?
3. What is this test intended to measure (e.g., past exposure, presence of a particular pathogen), and is that sufficient?
4. What are the characteristics of the test?
5. Given the quality of samples collected how well will this test perform?

6. Is the test readily available within my jurisdiction? If not, can I find laboratories that can conduct the test?
7. What are the resource requirements for conducting the test?
8. Are there legal or regulatory mandates that require certain tests?

These are some of the major considerations that need to be addressed when selecting a diagnostic test. We will consider them in more detail below.

It is important to note that the OIE has specific information for OIE-listed pathogens in "Manual of Diagnostic Tests and Vaccines for Terrestrial Animals: ([Terrestrial Manual Online Access - WOAAH - World Organisation for Animal Health](#)) and the "Manual of Diagnostic Tests and Vaccines for Aquatic animals ([Aquatic Manual Online Access - WOAAH - World Organisation for Animal Health](#)). However, these resources are limited with regard to testing of wildlife species.

2. Auxiliary Information

In some instances auxiliary information may be available for a given outbreak to aid in diagnostic test selection. For instance, if carcasses or samples have been sent to a pathologist, these scientists, based on their examinations, can provide key knowledge about potential pathogens or diseases that may have led to the outbreak event based on their gross findings during necropsy. For example, histopathology is one particularly useful technique that pathologists can use to help determine what diagnostic tests may be most useful to conduct. Histopathology, or the microscopic examination of tissues, allows the pathologist to assess abnormalities at the cellular level. Particular changes noted in the tissue, such as degree, location and type of inflammation, presence of necrosis, fibrosis, or other cellular processes, can suggest a particular infectious or toxic cause of mortality. In the case of bacterial, fungal and parasitic diseases, infectious organisms can often be seen in the tissue section. This is especially useful as a corollary to diagnostic tests in order to associate an infectious agent with a particular tissue lesion and implicate it as a cause of disease. One caveat regarding the use of histopathology is the need for fresh tissues; due to artifacts created by decomposition, histopathology is not useful for decomposed samples. However, this does not preclude the examination of decomposed samples by other methods.

3. Test Validation

The OIE defines test validation as a process that determines the fitness of a test, which has been properly developed, optimized and standardized, for an intended purpose. A general discussion of test validation can be found at [https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/2.02.00 INTRODUCTION.pdf](https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/2.02.00_INTRODUCTION.pdf), and will not be discussed here in detail. However, validation of a test provides the assurance that the test will correctly and reliably identify the pathogens or diseases of concern. Test validation is an intensive and rigorous process and therefore, requires significant resources. As a result, diagnostic tests tend to be validated on only specific samples (e.g., serum) from a few species of animals. This may be problematic for determining potential pathogens or diseases involved in an outbreak in wildlife because generally no validated test exists for wildlife species. A detailed discussion of test validation with regards to wildlife species can be found at [https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/2.02.07 WILDLIFE.pdf](https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/2.02.07_WILDLIFE.pdf).

4. Test Target

The target, or what a test is intended measure, is another critical consideration in determining diagnostic test selection. Particularly, in the absence of a validated test, it is recommended to select diagnostic tests that are less likely to be affected by the species involved in the outbreak. For example, indirect tests that target a host immune response (e.g., ELISA) or measures past exposure would be less desirable than a direct test that attempts to detect the presence of a pathogen (e.g., PCR). Some simple guidelines are presented for illustration in the table below.

This table provides a few examples of potential tests and their likelihood of being affected by host species. This table was taken from an OIE training course on surveillance techniques

http://www.oie.int/fileadmin/Home/eng/International_Standard_Setting/docs/pdf/WGWildlife/A_Training_Manual_Wildlife_2.pdf, which contains relevant information with regards to outbreak investigations.

	Less likely to be affected by host species	Intermediate	More likely to be affected by host species
Tests for pathogens	Direct identification (e.g., parasites)	Culture for viruses ³	
	Culture: bacteria, fungi, protozoa		
	PCR - matrix dependent ⁴		
	Immunohistochemistry		
	Chemical analysis (e.g., toxicology)		
Tests for Antibodies or Immune Response	Virus neutralization		Serology tests: ELISA
	Blocking (competitive) ELISA		Antigen skin tests (TB)
Other	Genomics	Brain	Metabonomics Transcriptomics
		Cholinesterase ⁵	

Of interest are emerging fields of genomics and metabonomics. Genomics is defined as: "a branch of biotechnology concerned with applying the techniques of genetics and molecular biology to the genetic mapping and DNA sequencing of sets of genes or the complete genomes of selected organisms, with organizing the results in databases, and with applications of the data⁶. This technology can be useful in determining causal biological agents associated with outbreaks. For example, it has been used successfully to track the spread and effects of reassortants of avian influenza viruses. Similarly, metabonomics is defined as "the quantitative measurement of the dynamic, multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification⁷. In short, metabonomics measures the complete set of metabolites present in an organism or associated biological samples, and examines changes in these due to perturbations such as infection or disease. Metabonomics is closely related to metabolomics, which is defined as the "systematic study of the unique chemical fingerprints, in the form of small-molecule metabolite profiles, that are left by specific cellular reactions"⁸. Although there is some disagreement on the differences between these two fields, metabolomics generally focuses on metabolites created at the cellular or organ level under normal endogenous metabolism; whereas, metabonomics focuses on

³ Some viruses can be cultured in standard cells culture or chicken embryos, but others require their specific host cells in which to grow

⁴ Problems can arise using PCR on some samples from some species (e.g., ruminant feces)

⁵ This is used as a screening test for poisoning by organophosphate and carbamate insecticides. Background levels are variable across species.

⁶ As defined by: <http://www.merriam-webster.com/dictionary/genomics>

⁷ As defined by: <http://medical-dictionary.thefreedictionary.com/metabonomics>

⁸ As defined by: <http://medical-dictionary.thefreedictionary.com/metabolomics>

the effects exogenous factors such as disease on metabolic profiles. Generally, metabonomic investigations have been limited to the human health field, but they may have potential use in the animal health arena too. Lastly, transcriptomics is the study of the RNA transcripts of a cell, tissue, or organism.

5. Test Characteristics

It is essential when selecting a diagnostic test to understand its characteristics or how well the test performs. If a test frequently produces incorrect results than it may be of limited use in outbreak investigations. An assessment of the likelihood of a test producing accurate results can be determined through the test validation process. Generally, the propensity for a test to return correct results is summarized by the characteristics of sensitivity and specificity.

- Sensitivity is defined as the probability of obtaining a positive test result given that the individual is truly infected.
- Specificity is defines as the probability of obtaining a negative test result given that the individual is not infected.

Example

Suppose we have experienced a die-off of 100 bighorn sheep (*Ovis canadensis canadensis*). Based on the gross necropsy results, the investigating pathologist has suggested that the cause of death is respiratory disease.



You are interested in determining if the causative agent is *Mycoplasma ovipneumoniae*, a bacterial agent known to cause respiratory disease- related die-offs in other populations of bighorn sheep. There is a bacterial culture test available that has been validated. The sensitivity of this test is 80% and the specificity is 90%. You have collected 100 oropharyngeal swab samples from dead bighorn sheep, and plan to use the bacterial culture test to assess the presence of *M. ovipneumoniae*. Given this information, how many false negative (FN) tests would you expect? In this example, we make an implicit assumption that if *M. ovipneumoniae* is the causative agent, all individuals will be infected. This is an important assumption that we will discuss further later. However, given this assumption we can quickly calculate the following:

$$F_n = 100 \times (1 - 0.80) = 20$$

This illustrates the impact of sensitivity on test results. We would expect that 20 of our 100 swab samples would falsely indicate the pathogen was not present.

This example also demonstrates that the underlying true prevalence affects our interpretation of test results too. In this case, specificity is not a concern because we assumed all individuals were infected. However, now let's assume that the true prevalence of *M. ovipneumoniae* is 80% in the dead sheep. We now can calculate both the number of false negatives as well as the number of false positives. We will use the following table to demonstrate that process.

		True infection status		Total
		+	-	
Diagnostic test result	+	$80 \times 0.80 = 64$	$20 \times (1 - 0.90) = 2$	66
	-	$80 \times (1 - 0.80) = 16$	$20 \times 0.90 = 18$	34
Total		80	20	100

Examining this table demonstrates that we would expect 16 of our swab samples

to provide false negative results (red highlighted cell), and 2 samples would provide false positive results (green highlighted cell). What this simple example clearly illustrates is that the characteristics of the diagnostic test directly affects the confidence we have in the reported results. In short, a positive diagnostic test result does not ensure that the targeted pathogen is present, nor does a negative test result definitively indicate absence of the pathogen. This also enforces why test validation is an important process, and why the lack of validated tests for wildlife is problematic.

Interestingly, despite sensitivity and specificity being independent of prevalence of a pathogen among individuals affected by an outbreak, true underlying prevalence also impacts diagnostic test interpretation. Let's repeat the above example, but now we will assume the true prevalence is 20%.

		True infection status		Total
		+	-	
Diagnostic test result	+	$20 \times 0.80 = 16$	$80 \times (1 - 0.90) = 8$	24
	-	$20 \times (1 - 0.80) = 4$	$80 \times 0.90 = 72$	76
Total		20	80	100

In this example, we would expect 4 of our swab samples to provide false negative results (red highlighted cell), and 8 samples would provide false positive results (green highlighted cell). So how does true prevalence in outbreak samples affect our interpretation of reported test results? In the real world, we do not know the true prevalence, rather we only know the reported test results. This is often called apparent prevalence. So in our first example, we would calculate our apparent prevalence as:

$$66/100 = 0.66.$$

In the second example, we would report an apparent prevalence of:

$$24/100 = 0.24.$$

In the first case we underestimate the true prevalence and in the second case we overestimate true prevalence...why? The answer lies in the fact that true prevalence, sensitivity and specificity interact to produce the observed positive and negative test results. This can be shown in a probabilistic form as follows:

$$\text{Pr}(+\text{test}) = \text{Pr}(+\text{indiv}) \times \text{Sensitivity} + (1 - \text{Pr}(+\text{indiv})) \times (1 - \text{Specificity}).$$

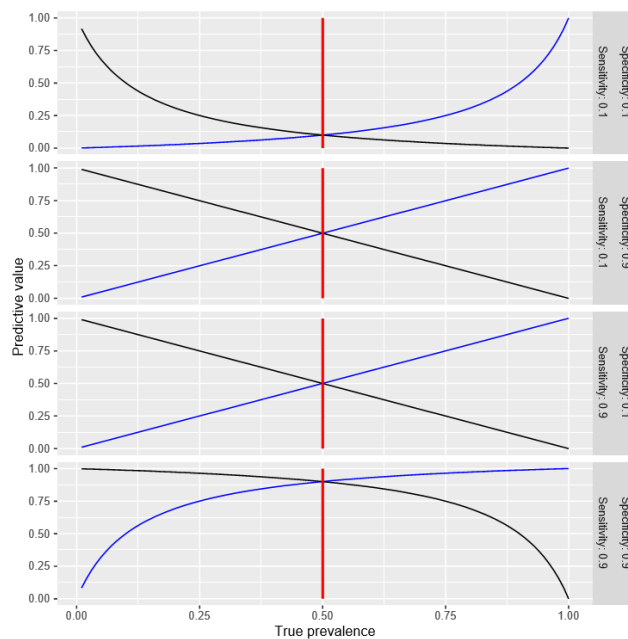
In words, the probability of obtaining a positive test results is equal to the probability an individual is truly infected or diseased (i.e., true prevalence) multiplied by the sensitivity of the test added to the probability of being truly uninfected or disease free multiplied by 1 minus the specificity. From this formula, it is clear that all three probabilities: true prevalence, sensitivity and specificity, interact to give rise to the observed test results. An interesting observation is that there is a trade-off between the probability a test-positive

animal is truly infected or diseased (i.e., known as the positive predictive value) and the probability of a test-negative animal truly being healthy (i.e., known as the negative predictive value). It turns out that these predictive values are jointly maximized at a true prevalence of 0.50. This is shown in the figure below where the positive predictive value is shown in blue and the negative predictive value is shown in black. The red line indicates where the predictive values are jointly maximized.

Why is understanding the relationship between true prevalence, sensitivity and specificity important in selecting diagnostic tests for outbreak investigations?

1. Sensitivity: It is important to have a highly sensitive test when the purpose of the diagnostic testing is to "rule-out: the presence of a specific agent or demonstrate freedom from a particular disease. A highly sensitive test means that there is a low probability that an infected/diseased animal would test negative (i.e., false negatives decrease).
2. Specificity: It is important to have a highly specific test when the purpose of the diagnostic testing is to "rule-in: the presence of a specific agent or disease. A highly specific test means that there is a low probability that a healthy animal would test positive (i.e., false positives decrease).

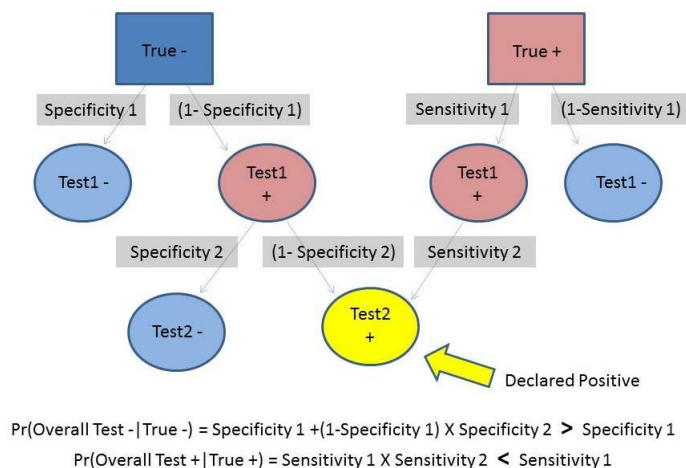
Therefore, sensitivity and specificity can guide diagnostic test selection for outbreak investigations or surveillance based on whether the objective of the activity is to "rule-in: or "rule-out: a particular agent.



3. True prevalence is valuable information because, given a test's characteristics, it directly impacts the likelihood of correctly classifying individual animals as healthy or infected/diseased. Diagnostic test choice can then be optimized based on what the true prevalence may be.

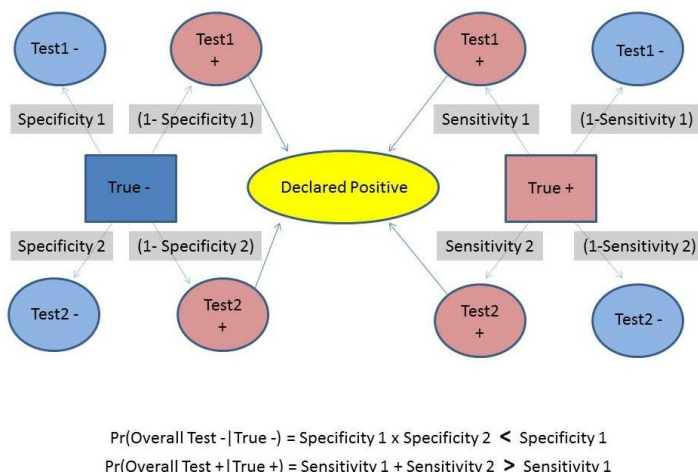
Reality Check. You might be saying to yourself, this is great information but in wildlife outbreak investigations I will likely not have a validated test nor will I know the true underlying prevalence: This is indeed true and the harsh reality of working with wildlife

species; however, the concepts discussed above can still be used based on qualitative knowledge about the system. For example, the very nature of various tests provide information regarding their relative characteristics. Thus, conducting a PCR test for a pathogen is generally more sensitive than a culture-based method. Additionally, it is often the case, during outbreak investigations, that we can assume if there is a single causative agent it should have a high prevalence among affected individuals. Thus, even in the absence of perfect knowledge, we may be able to select a diagnostic test that is best suited to our needs based on our understanding of the qualitative nature of the system and knowledge of how the various tests have performed under similar conditions in other systems. Another option when faced with imperfect knowledge about test characteristics and true prevalence rates is to conduct multiple tests for an agent. Multiple testing can be done serially or in parallel and can be conducted with different tests or the same test. Serial sampling is done by testing samples multiple times and declaring a positive detection only if all tests detect the agent. This is often done for surveillance activities with a cheaper "screening test: with high sensitivity being employed first, and test positive samples are then subsequently re-tested with a more expensive "confirmatory test: with high specificity. Serial sampling essentially increases the specificity and decreases the sensitivity of the testing protocol. The following graphic illustrates serial sampling and its effect on overall test characteristics.



In serial sampling, to be declared a positive test result, an animal must have tested positive (yellow oval) using the first test and then also using the second test. Therefore, truly positive animals have a probability of testing positive - to the product of the sensitivities of each test. Given each test's sensitivities are probabilities their product will be less than either test's individual sensitivity. Conversely, a truly negative animal will be declared test negative if either test 1 returns a negative result or if test 1 returns a positive result and test 2 returns a negative result. Thus, probability of testing negative given you are negative is the sum of their specificity of test 1 and the product of (1- specificity of test1) and specificity of test 2. This sum must be greater than either test's individual specificity.

Parallel sampling involves testing samples with multiple tests and declaring a positive detection if at least one of the tests returns a positive result. Parallel sampling increases sensitivity at the expense of specificity. The following graphic illustrates serial sampling and its effect on overall test characteristics.



In parallel sampling, to be declared a positive test result (yellow oval), an animal must test positive to either test 1 or test 2. Truly positive animals have a probability of testing positive -to the sum of each individual test's sensitivity. Thus, sensitivity of the parallel test design must be greater than the individual sensitivities of each test. For truly negative animals, to be declared test negative they must be declared negative by each test, which implies the probability of returning a negative test result is the product of each test's specificity. This product will be less than the individual test's specificity.

In serial and parallel sampling, we rely on the overall rules of probability to understand the characteristics of the resulting test; however, the actual values of the true prevalence, sensitivity and specificity remain unknown. This may be undesirable, particularly when an estimate of true prevalence of an agent within individuals sampled during an outbreak investigation is required. In this case, we can take advantage of techniques used in ecology for species distribution modeling known as occupancy modeling⁹. Under a few simplifying assumptions (i.e, test sensitivity > (1-test specificity)), these techniques can be directly applied to diagnostic testing for an agent where site occupancy is analogous to presence of the agent in a sample; occupancy probability -true prevalence; detection probability - test sensitivity; and false detection probability - 1- specificity. The benefit of this approach is the ability to directly estimate true prevalence, sensitivity and specificity. However, there is no "free lunch", and to employ this technique each sample must be tested multiple times (i.e., 2) using the same test. Therefore, there are additional diagnostic costs associated with this technique.

In summary, it is important to understand the characteristics of the various diagnostic tests available as well as the true prevalence rate of the agent for which the test is being applied. However, in general for most wildlife outbreaks this information will be lacking. In these circumstances, general characteristics about the various tests, the test target, and educated assumptions about underlying prevalence rates based on expert knowledge or past experience in similar outbreaks, can be useful proxies for complete knowledge. Additionally, multiple tests can be applied to increase sensitivity or specificity based on the objectives of the investigation, or ecological occupancy modeling can be used to estimate

⁹ Ecology. 2006 Apr;87(4):835-41.

the parameters of interest.

6. Sample Quality

The condition or state in which animals or their carcasses are can play a significant role in what diagnostic tests can be used during an outbreak investigation. In fact, if carcasses are too autolyzed diagnostic testing may not even be possible. In general, collecting biological samples from moribund animals or recent mortalities provides the greatest amount of choices of diagnostic tests. However, if fresh samples cannot be collected, direct tests that target the pathogen, particularly molecular-based techniques that do not require a viable agent, may be the most useful. If possible, a pathologist should be consulted prior to sample collection to ensure the full breadth of potential tests are known and proper samples can be obtained.

A second consideration is the handling of the sample once it has been obtained. Handling can directly impact sample quality, and may impact the usefulness of a diagnostic test. Handling involves many different aspects including temperature, transport medium, length of time from collection to diagnostic testing, container, etc. For example, in studying bighorn sheep respiratory disease it has been documented that for bacterial culture tests, the time from sample collection to diagnostic testing and the associated temperature fluctuations that may occur in that time period, can result in overgrowth of non-target species and negatively impact the detection of target *Pasteurella* species. Therefore, it is also imperative to consider the conditions and how samples can be reasonably handled in the field prior to selecting a diagnostic test.

7. Test Availability

The availability of a desired diagnostic test is another clear consideration. It is possible that the preferred test may not be readily available, and in these situations it may be necessary to see if other jurisdictions have the capacity and willingness to partner. Such partnerships provide an excellent opportunity to create collaborations and regional networks of partners that can support each other in conducting wildlife outbreak investigations. The OIE laboratory and collaborating centres network are another potential resource. The OIE has established particular laboratories, based on their expertise, as OIE Reference Laboratories for specific pathogens. These laboratories can be invaluable resources with regards to testing for particular pathogens and interpretation of test results. Information regarding these laboratories can be found at [Expertise Network - WOA - World Organisation for Animal Health](#). However, there may be some challenges in shipping samples to OIE Reference Laboratories, including inter-country permitting, that should be investigated to determine if this is a viable option. If no palatable option can be found, a different diagnostic test may need to be used even if it is less preferred.

8. Resource Requirements

Resource requirements are an important aspect of selecting a diagnostic test. Some diagnostic tests (e.g., genomic tests) can be expensive to run, particularly for a large number of samples. Establishing the availability of funding for diagnostic testing in an outbreak investigation may clearly limit the choice of diagnostic tests. Cost is not the only resource consideration. Some tests require specialized equipment for collection (e.g., particular media), or require skilled personnel. Although perhaps painfully obvious, a requisite prior to selecting a diagnostic test is a clear understanding and assessment of availability of the necessary resources to successfully implement the test.

9. Legal Mandates

A final consideration is whether there are specific legal mandates associated with testing for a pathogen or agent. For example, within the OIE Terrestrial <https://www.woah.org/en/what-we-do/standards/codes-and-manuals/terrestrial-code-online-access/> and Aquatic Codes <https://www.woah.org/en/what-we-do/standards/codes-and-manuals/aquatic-code-online-access/>, tests are prescribed for specific pathogens "to assure the sanitary safety of international trade in terrestrial animals and aquatic animals, and their products: <https://www.woah.org/en/what-we-do/standards/codes-and-manuals/terrestrial-manual-online-access/>. Thus, to demonstrate an outbreak is not caused by a specific OIE- listed pathogen these particular tests should be conducted. The terrestrial and aquatic manuals described earlier provide further guidance in specific application of tests.

10. Examples

We conclude this discussion by examining several different diagnostic techniques used in wildlife outbreak investigations: culture techniques and viral diagnostics. The intent of these examples is to describe some of the unique challenges involved in employing several diagnostic techniques used for wildlife samples, and illustrate many of the considerations discussed above.

Culture Techniques. Culture techniques are frequently used to assist with diagnosing disease. In many cases, culture analyses are relatively inexpensive, require little in the way of specialized equipment, and can yield results in several days. The technique is particularly important when isolates of a pathogen are needed for downstream applications (e.g., strain typing, genetic analyses, etc.) and for instances in which there is no suspected cause of a mortality event, or pathogen-specific tests (e.g., PCR) fail to identify a causative agent. White-nose syndrome (WNS) in bats provides a perfect example of the importance of culture techniques in identifying a causative agent. When WNS was discovered in 2007, the causative agent was unknown. Thus, there were no molecular-based tests (e.g., specific PCR assays) that could be used to screen for the pathogen that causes the disease. Instead, it was through culture analysis that a single fungus was found to be consistently associated with WNS. Obtaining living cultures of the fungus was also essential for downstream applications such as laboratory infection trials to confirm its pathogenicity, development of PCR assays to more quickly screen for the pathogen, and genetic analyses that demonstrated the fungus was introduced to North America from Eurasia.

However, culture analyses can also have major limitations. Specifically, culture techniques require that the pathogenic microorganism is capable of being grown in the laboratory, that the correct culture conditions are used; the pathogen is viable in the sample being tested; and the sample is not contaminated with other microorganisms, which may confound the ability to recover the pathogen of interest. In most cases, culture techniques must be combined with other analyses (such as histopathology) to generate an accurate diagnosis because samples will be contaminated with microorganisms that are not actually the cause of the disease. In the case of WNS, the causative agent took multiple attempts to isolate because the fungus only grows at cold temperatures (non- standard laboratory growth conditions) and is often difficult to isolate from the many faster-growing fungi that are typically present in the sample. For example, the fungus *Mucor* is frequently cultured from the wings of bats with WNS. Some species of *Mucor* are considered pathogens, but this fungus is clearly not the cause of WNS.

Viral Diagnostics. A large number of viral families are known or suspected to infect wild animals. Due to their independent evolution history and replication strategies, there is no conserved motif or nucleotide sequences that can be used as a universal detection target that would be the equivalent of the 16S/18S ribosomal RNA in bacteria and fungi. Instead

viral diagnosis may range from a broadly family-specific screening assay (e.g. coronaviridae) to a strain-specific test (e.g. MERS) depending on the diagnostic needs. Thought should be given as to the collection of appropriate samples. Agents that have a viremic phase, might have high circulating titers during peak viremia, but the concentration of virus might drop precipitously in face of a mounting immune response. Some viral agents have a pronounced tissue tropism, and collection of the appropriate organ will affect the likelihood of successful identification. The choice of culture systems will affect the ability to recover (and ultimately identify) the viral agent. Some viruses are markedly host and tissue specific, while sometimes others in the same viral family are not. Depending on the viral replication mechanism, some viruses require cells to be actively replicating while others do not. In general, culture systems and conditions should mimic the reservoir host as much as possible. Providing a variety of culture systems and diagnostic tests capable of addressing wildlife health needs is an ongoing challenge.

VI. Study Design

1. Purpose

The intent of this section is to highlight some of the key considerations that should be addressed prior to conducting an outbreak investigation or surveillance work; however, we will frame our discussion in terms of outbreak investigations. It is often the case during outbreaks that there is a need for a quick and immediate response; however, this is not a justification for failing to clearly lay out a suitable study design prior to initiation of activities. Failure to address design issues may result in an inefficient response or, in the worst-case scenario, the inability to meet the objectives of the investigation. We conclude our discussion with a focus on study design because of its importance to successful outbreak investigations, and to conclude with an emphasis on this foundational topic.

2. Objectives

The first step in an outbreak investigation is determining what are the objectives of the investigations. The objectives are the foundation upon which the entire investigation is built, and clearly stated objectives not only predetermine the necessary activities, but also provide guidance during an investigation when questions arise. Some key questions that can help formulate objectives are:

1. Why am I conducting an outbreak investigation?
2. What do I hope to learn from this investigation?
3. Who are the stakeholders involved?

Although, the first question may seem trivial, nevertheless it is foundational. For example, is the investigation being conducted to ensure that the die-off is not associated with a OIE-notifiable pathogen/disease; is it being conducted to determine if there is a newly emerging disease; is it being conducted as part of routine surveillance activities; etc? There are significant implications regarding study design depending on how this question is answered.

The second question expands upon the first, and also helps in creating an appropriate study design. For example, is the intent of the investigation: to learn whether a specific pathogen is present in individual animals involved in the outbreak; to estimate the prevalence of a pathogen; to determine potential risk factors; to determine spatial extent, etc. This information will determine how many individuals will need to be sampled, how sampling will be conducted and over what scale.

The final question is useful to identify groups of individuals to whom information concerning the outbreak may need to be disseminated or solicited. Stakeholder involvement can assist in collecting information on further outbreaks that may not have been detected otherwise. Additionally, early involvement of stakeholders can be beneficial to help understand the potential societal risk posed by the outbreak, and may define the type and extent of action needed for an investigation. Lastly, identifying stakeholders lays the groundwork for developing an effective public communication strategy.

3. Sampling Design

Once clear objectives have been established, the next step is to determine what is the metric of interest, and how many and in what manner should samples be collected. There are numerous different sampling designs that can be applied depending on the specific outbreak(s) being investigated. We detail a few common methods here.

a. Metrics

There are a variety of metrics that can be used to summarize and analyze information collected during an outbreak investigation depending on the objectives. Understanding how the data will be analyzed is necessary to determine other aspects of a study's design. We describe three of the most common metrics.

Freedom From Disease. Establishing the outbreak is not associated with a particular pathogen (e.g., OIE-notifiable pathogen/disease) involves collecting presence/absence data from individual animals associated with the outbreak. This means each individual or a subsample of individuals involved in the outbreak are tested for the pathogen of interest, and either are determined to be free of the particular pathogen or not. If presence (i.e., the pathogen is identified) is established within an individual, then clearly the individuals are not disease-free. If this was the sole purpose of the investigation, no further work need be conducted. However, if the pathogen is determined to be absent from all tested individuals, it is possible to assess what the underlying infection probability of the pathogen could be for a specified confidence-level, and still be undetected during testing. This infection probability (i.e., design prevalence) may be known if the exact sample size (described below) necessary to achieve a desired confidence for a specified design infection rate was collected; however, it is common the number of individuals sampled will fall short or exceed this target sample size. In these cases, within a Bayesian statistical framework and given the number of individuals sampled, it is possible to estimate the underlying infection rate and its upper 95% credible bound. The 95% upper credible bound is the value at which there is a 95% probability the true infection rate is below, and is generally the value of most interest. A suitable reference describing this technique and how to incorporate auxiliary information can be found at <http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0089843>.

Prevalence. Prevalence is one of the most common disease metrics used to measure the intensity of a pathogen/disease. It is the proportion of individuals involved in an outbreak that have a disease/pathogen, and characterizes how wide-spread an infection is within a population. It is usually based on a cross-section of the population, and if based on a single outbreak event arises from a "snap-shot: in time. However, in outbreak investigations it may be difficult to determine over what length of time the prevalence metric applies because the outbreak may have occurred over an extended time period prior to it being discovered. Prevalence and its associated measure of precision are easy to calculate, and more sophisticated analyses can be conducted to examine risk factors, spatial and temporal relationships.

Incidence. Incidence is a much less used metric in wildlife disease. Incidence is the rate of new cases within a population for a given time period, and represents the risk of infection. One of the main reasons incidence is rarely employed is because it requires frequent and regular monitoring of the population to establish the number of new cases that have occurred since the previous monitoring period. This can be quite difficult for wildlife populations that are often able to move in and out of a region, and can succumb to a disease without being observed. However, there are instances that it can be employed if the objectives of the investigation require it. For example, frequent monitoring of a small pond for amphibians that have died due to chytridiomycosis. Like prevalence more sophisticated modeling endeavors can be undertaken to elucidate various risk factors and other relationships.

b. Sample Size - Individual Animals - Single Site

Interestingly, sample size calculations are the norm for establishing the amount of effort required for surveillance activities, but often are neglected when conducting outbreak investigations. This is unfortunate because it may prohibit meeting objectives of the investigation. With clear objectives, sample size calculations can be used to estimate the amount of sampling effort required.

The first step in estimating necessary sample sizes during an outbreak investigation is to clearly define the population of interest. This may be quite simple, for example bats in a hibernaculum, or it may require some "hard-thinking. for example an investigation of migratory bird mortalities involving several species. Regardless, the importance of this step cannot be over-emphasized because it determines the sampling frame, which provides the context for sample size calculations. For example, if I say, "I want to detect a 1% prevalence of pathogen X with a 95% confidence, but do not provide a definition of the population then this statement has no probabilistic meaning. In other words, this means that the 1% prevalence requirement inherently assumes there is a population to which the prevalence applies, and if the population definition is lacking it has no interpretation. Defining the population also is important in determining the spatial extent of sampling (described below) that needs to be conducted, and is necessary when reporting results.

The second step is to determine how precise of an estimated disease metric (e.g., prevalence) or how much confidence of freedom from disease is required. This information is required for any analysis to determine sample size. If an extremely precise estimate is required or a high-level of confidence of freedom from disease is sought, there will be an associated increase in sample size requirements. Therefore, there will be a trade-off between effort and associated costs and the precision/confidence obtained.

Once a population is defined and level of precision/confidence is specified, estimates of necessary sample sizes can be calculated. The second cycle of this series of trainings (http://www.oie.int/fileadmin/Home/eng/International_Standard_Setting/docs/pdf/WGWildlife/A_Training_Manual_Wildlife_2.pdf) addressed the topic of sample size calculations for detecting a pathogen as well as estimating the prevalence of a pathogen for a surveillance program. The same techniques are applicable for outbreak investigations, and we will refer interested parties to that reference. We would like to note that a useful tool for estimating sample sizes needed for surveillance programs or outbreak investigations is freely provided by AusVet¹⁰.

c. Sampling Geographical Units

A common objective of outbreak investigations is to determine the spatial extent of an outbreak or the extent to which a population is affected across its range. In some situations this may be relatively easy. For example, a frog mortality event in a small, isolated, high-alpine lake. Other times it may be much more difficult (e.g., die-off of white-tailed deer due to epizootic hemorrhagic disease¹¹). In the latter case, to rigorously assess the spatial extent of the die-off, a census or probabilistic sample of geographical sites may need to be taken because the outbreak event is not limited to a single location. Geographical sampling considerations may also arise when an outbreak happens in a discrete unit (e.g., a lake), but the unit is too large to completely survey for dead individuals. In this situation probabilistic sampling is also important to allow inference to be made to the entire event.

The first step in sampling geographical units is to define the spatial extent of interest. This is analogous to defining the population for sampling individual animals as described above, and the same justification applies. Once the spatial extent is defined, the next step is to determine whether the entire spatial extent will be surveyed or will it need to be sampled. Lastly, if sampling is required, it must be decided whether the spatial extent will be aggregated (e.g., broken into discrete grid cells) and the method of aggregation (e.g., grid of cells of equal size) or will it be treated as a continuous surface. Aggregation is the most common approach, and will be the major focus of our discussion. Once these design questions are answered a sampling design can be created. Generally, these designs can be thought of as either one-stage or two-stage sampling designs. First, a sample of geographical units are selected, and subsequently a census (one-stage) or a second sample (i.e., of individuals; two-stage) is conducted within the selected geographical units. There are many probabilistic designs that can be used. We will describe several common choices focusing on selecting geographical units, but similar procedures can be implemented at the second stage if individual animals cannot be sampled completely within the selected geographical units.

Simple random sample. The simplest probabilistic sampling design is to draw a random sample¹². If the data are aggregated this can be accomplished by assigning each grid cell an equal probability of being selected. A random sample can then be drawn using many software programs. For example, in Excel the

¹⁰ <http://epitools.ausvet.com.au/content.php?page=home>

¹¹ http://www.michigan.gov/dnr/0,1607,7-153-10370_12150-26647--,00.html

¹² In our sampling designs we will sample without replacement. This means that a geographical unit can only be selected once.

following steps can be used to select a sample of size "n: from "N: total grid cells.

1. Open a spread sheet in Excel.
2. Create a column of numbers from 1 to N in the first column.
3. Insert two empty columns to the right of your data.
4. Type - RAND() into your first empty cell in the second column.
5. Copy the Rand() formula and paste it all the way down your second column. This will generate a column of random numbers.
6. Highlight the whole second column of random numbers and copy it.
7. Paste the random values in the third column using "Paste Special;, and the values only option.
8. Sort all 3 columns in ascending order of random values in column 3.
9. Numbers in the first column found in rows 1 to n represent the grid cells to sample.

If the data are treated as continuous, "n: random points must be generated within the spatial extent. There are several specialized software packages available to easily facilitate this including the statistical program R (described below) as well as many different Geographic Information Systems software packages. If the spatial extent is rectangular, the following algorithm can be used in Excel:

1. Open a spread sheet in Excel.
2. Create a column of n random numbers in the first column.using -RAND() function.
3. Highlight the whole first column of random numbers and copy it.
4. Paste/overwrite the random values in the first column using "Paste Special;, and the values only option.
5. Determine the maximum and minimum X and Y values of your spatial extent.
6. Calculate the difference between the maximum and minimum values for X and Y.
7. In the first cell of the second column of the spreadsheet enter the following formula: $A1*(Xdiff)+Xmin$, where Xdiff is the difference between the maximum and minimum X values, and Xmin is the minimum X value of the spatial extent.
8. Copy the formula down column 2 until you reach row n.
9. In the first cell of the third column of the spreadsheet enter the following formula: $A1*(Ydiff)+Ymin$, where Ydiff is the difference between the maximum and minimum Y values, and Ymin is the minimum Y value of the spatial extent.
10. Copy the formula down column 3 until you reach row n.
11. Column 2 now contains the X coordinate and column 3 the Y coordinate of the n random spatial locations within the rectangular spatial extent.

Stratified Random Sample. A stratified random sample is used when a

random sample is desired, but there are various non-overlapping strata within the spatial extent that are important and affect the outbreak in some way. Generally, to employ this technique independent random samples of equal size (i.e.,

$\frac{n}{h}$) are drawn from each stratum using the procedures described above.

For example, suppose we have human-developed and undeveloped strata within our spatial extent, and we expect fewer animals occur in the human-developed regions. We may be interested in assessing differences in the intensity of the outbreak between these strata. In this case, stratified random sampling is a suitable sampling design. It is important to note that there are several different possible means to allocate samples size between strata beyond equal sample sizes. A statistician should be consulted to assure optimal sampling designs are implemented.

Unequal Probability Sample. In the previous sampling approaches, the probability of a geographical unit or location being selected has been equal across all units or locations. However, unequal probability sampling designs, with varying probabilities of selection, may also be appropriate and are often desirable in some situations. Unequal probability sampling is used when there is auxiliary information available to bring to bear on the problem. This information can then be used to improve (i.e., decrease variability) our estimates of disease metrics, or increase our likelihood of detecting a pathogen if it is present within our spatial extent. For example, suppose we believe that the probability of detecting a pathogen is related to the density of the host on the landscape. Additionally, we have spatially-varying, host density estimates across our spatial extent of interest. If we have our aggregated our spatial extent, we can estimate the density for each cell. We can then use the cumulative-size method to estimate the sampling probability for each grid cell. The procedure to generate sampling probabilities and select grid cells using those probabilities in Excel is as follows:

1. Open a spread sheet in Excel.
2. Create a column of numbers from 1 to N in the first column.
3. In the second column, enter the host density estimate for each grid cell from 1 to N. Be sure the density estimate corresponds to the grid cell number in the adjacent cell in the first column.
4. In the third column, type the following formula into the first cell: $B1/\text{sum}(\$B\$1:\$B\$N)^{13}$. This is the sampling probability.
5. In the first cell of the fourth column enter the following formula: $-C1$.
6. In the second cell of the fourth column enter the following formula: $-\text{SUM}(\$D1,C2)$.
7. Copy this formula down to row N.
8. In the first cell of the fifth column enter the following formula: $-\text{RAND}()$.
9. Copy this formula down to row n.
10. Highlight the whole fifth column of random numbers and copy it.
11. Paste the random values in the sixth column using "Paste Special:", and the values only option.
12. Determine the row containing the largest value in the fourth column < the random value in the first cell in the sixth column. This row number is the

¹³ Replace N in the formula with the actual total number of cells.

- first grid cell selected. Record it.
13. Find the cell in the second column that corresponds to the row number selected in step 12, and set the value in that cell to zero.
 14. Now repeat steps 12 and 13 until all n grid cells have been selected. Note a cells that are previously selected and their density values set to zero are not available again for selection.

Selecting samples using the above procedure is clearly only feasible when n is small because it is slow and tedious. Generally, other software packages can be used and are more efficient. We present simple code demonstrating how to select an equal and unequal probability sample using the open-source statistical program R¹⁴. For this coding example, we assume a comma separated values (.csv file) has been created that contains the grid cell identifiers in column 1, density estimates for each cell in the second column, and the sampling probabilities in the third column (i.e., file created using steps 1-4 above).

```
setwd("C:\\temp") #set the working directory
input<-read.csv("filename.csv") #import data in .csv format

output.equal<-sample(1:nrow(input), n,
  replace = FALSE, prob = NULL) #equal prob. sampling
output.unequal<-
sample(1:nrow(input), n,
  replace = FALSE, prob = input[,3]) #unequal prob. sampling

output.equal #view results
output.unequal #view results

write.csv(output.equal, "eql.csv") #write results to .csv file
write.csv(output.unequal, "uneql.csv")
```

It is clear that it is simple to conduct these sampling procedures using R. Other software packages also have similar capabilities.

Another common use for unequal sampling probability designs is to use the spatial proximity of a grid cell to the location of a known outbreak site. Thus, grid cells close to the outbreak are sampled with a high probability, and that probability decreases with increasing distance from the site.

If the spatial extent of interest is not aggregated unequal sampling probabilities can be used to generate geographic locations; however, these techniques are more involved and a statistician should be consulted. Therefore, this topic will not be addressed here.

Convenience Sampling. Convenience sampling in outbreak investigations is the most common type of sampling, but unfortunately the least rigorous. Convenience sampling does not rely on a probabilistic underpinning to choose geographical units or locations, rather investigators use their own judgment to decide what grid cells or locations are selected. The decision is generally based on logistical considerations such as ease of access. Although this sampling approach is the easiest to implement, it is problematic for several reasons. First, it is not repeatable, which is a tenet of scientific investigations. Secondly, it can create biased samples that lead to incorrect inferences. Lastly, it can severely limit the inference that can be made from the collected data, unless strong and generally invalid assumptions are made. We do not recommend

¹⁴ <https://cran.r-project.org/>

convenience sampling, and suggest a probabilistic sampling scheme be used whenever possible.

Sample Size. Determining the necessary sample sizes when geographical considerations are incorporated into probabilistic sampling designs can be complex because for a given cost there are trade-offs between the number of geographical units sampled and the number of individuals sampled within a geographical unit. In general, the following information or an approximation will be minimally necessary:

1. Desired precision or confidence of the investigation.
2. Test sensitivity and specificity.
3. Costs associated with sampling geographical units and individual animals.
4. Variability across geographical units.
5. Variability across individual animals within geographical units.

For one-stage sampling designs, tools used for determining sample sizes for individuals from a single-site, as previously described, can be used. Additionally, AusVet provides some tools for calculating sample size when the objective is to demonstrate freedom from a disease/pathogen using a two-stage design. The tools are set up for sampling individuals across multiple herds, but they can also be used for sampling geographical units by recognizing geographical units can be used in place of individual herds.

However, in general, when using two-stage sampling designs for estimating disease intensity metrics during outbreak investigations, it will be necessary to consult a statistician because the required sample sizes of geographical units and individual animals within units will require calculating variances and will often be a computer optimization problem. Specifically, finding the optimal solution may require writing custom computer code to maximize the precision while minimizing the costs, and will need to be tailored to each outbreak investigation.

VII. Miscellaneous Considerations

Some additional considerations, relevant to outbreak investigations, is the fact that multiple pathogens and multiple species may be involved in an outbreak. Multiple pathogens are often isolated from individuals during an outbreak, and some may be commensal. This introduces an added layer of complexity when the objective is to establish a causative factor. In these cases, techniques such as histopathology may be particularly useful to help eliminate some pathogens as playing a role in morbidity or mortality. There should also be a recognition that no single pathogen may be solely responsible, but rather it may be the coinfection of multiple pathogens that ultimately gave rise to an outbreak. In the latter case, it may be necessary to lay out a study design that includes collecting and sampling healthy individuals from the same geographic region to permit modeling of the risk of morbidity/mortality as a function of the microbial flora.

Multiple species also increase the complexity of the study design. This can be particularly true in large avian outbreaks where many species may be commingling and are susceptible to cross-species transmission. In these instances, it is critical to establish what the population of interest is prior to initiating an investigation. For example, is the population of interest all

species involved in, or is there interest in each species individually? Answering this question, will determine if the study design aspects described should be applied individually or across the species involved.

VIII. Additional Resources

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World Organisation for Animal Health. 2015. Training manual on surveillance and international reporting of diseases in wild animals. (http://www.oie.int/fileadmin/Home/eng/Internationa_Standard_Setting/docs/pdf/WGWildlife/A_Training_Manual_Wildlife_2.pdf).

IX. Table Top Exercises

Wildlife outbreak investigation in the "Dominion of Atlantis":

(Note: This country does not exist)

Scenario 1

You are the lead wildlife epidemiologist for the Ministry of Health in Dominion of Atlantis.

You have received a report from a civilian, reporting the finding of 5 dead animals on her property. She is unsure of the species, but believes it is a type of antelope. The civilian found the dead animals when she drove the perimeter of her property on an All Terrain Vehicle this morning. She did not see any obvious signs of trauma, and is not aware of large predators in the area. She is very upset about the dead animals, and wants to have someone come out to remove them and figure out what happened. The civilian also lets you know she is planning to call the local media to let them know.

You record information from the civilian as quickly as you can, and get her contact information and location.

Within 1 hour of the first phone call, a story breaks on the local radio station about the findings from the first location, and another report comes in from a retired high school science teacher who has just returned from a hike. He reports a similar, disturbing scene from his hike in the upper regions of the Island of Atlantis where he found 10-12 deceased antelope. The teacher hikes this area 4-5 times per week, and frequently sees animals in the area. He is certain they were not dead 48 hours ago. The teacher offers to help your crew to find the area of concern, as it is a little off the main path. He also offers to help with work that may be necessary.

Your office is located in the capitol of Atlantis, in the main city of Big-town. Detailed information about Atlantis, the Atlantian antelope, and maps are provided in Appendix A to help with this scenario.

Activity

In small groups, review the material on outbreak investigation and steps necessary to prepare to respond.

Outline, in the spaces provided below, what your group would need to respond to this outbreak in the first 24 hours after receiving the report.

1. Who are the personnel you would contact for your response? What logistics are you considering when preparing for your initial outbreak investigation? Discuss and prioritize what you would need to get your personnel to respond to and conduct an investigation of an outbreak. This question applies to the details of mobilizing your team.
2. What are some considerations you will have in order to prepare for proper sample collection, handling, and laboratory testing?
3. What is your plan for data collection at your arrival to the outbreak site?
4. Describe your plans for initial disease control if it is needed.

Fun With Numbers Exercise - Individual exercise to provide an opportunity to better understand these concepts, complete the following tables using the information provided below.

Exercise A

You are investigating a die-off of 1000 bighorn sheep. You have diligently sampled each animal and have collected 1000 oropharyngeal swab samples. You know the true prevalence of *M. ovipneumoniae* is 25%, and the sensitivity of your test is known to be 80% and the specificity is 90%. Using that information complete the table below.

		True infection status		Total	Predictive values
		+	-		
Diagnostic test result	+				
	-				
Total				1000	

Note: Positive predictive value = Number of infected animals correctly classified/Total number of test positive animals; Negative predictive value = Number of healthy animals correctly classified/Total number of test negative animals

Calculate apparent prevalence rate in this example.

Exercise B

Now repeat these calculations with a true prevalence of 75% and the sensitivity of your test is known to be 80% and the specificity is 90%.

		True infection status		Total	Predictive values
		+	-		
Diagnostic test result	+				
	-				
Total				1000	

Calculate apparent prevalence rate in this example.

Exercise C

Describe in your own words the effect of true prevalence on apparent prevalence estimates and predictive values based on your results in Exercise A and B.

Group Exercise

Discuss among group members how information on test sensitivity, test specificity and true prevalence is useful for selecting diagnostic tests in an outbreak investigation.

Can you think of any means to increase predictive values of a test?

Scenario 2:

Your initial investigation of the antelope mortality event described in Scenario 1 indicates that the antelope likely died from some type of acute disease. Due to the high societal value associated with the Atlantian antelope, you have been instructed to conduct a "full: outbreak investigation for the population of Atlantian antelope on the Island. In particular, your supervisor directs you to determine the geographic extent of the outbreak on the island. However, he indicates that resources are limited and surveying the entire Island is not possible. Detailed information about Atlantis, the Atlantian antelope, and maps are provided in Appendix A to help with this scenario.

Question 1 (Group):

Establish objectives for your outbreak investigation.

What metric(s) will you use to characterize the disease outbreak? Why did you choose this metric(s)?

Question 2 (Group):

Based on the information you have been given, describe how you would collect data to determine the spatial extent of the outbreak on the Island.

How would you sample antelope populations across Atlantis?

Why did you choose this sampling design?

Question 3 (Group):

Now suppose that you fell ill and were not able to lead the outbreak investigation. Your colleague was assigned to fill in by your supervisor during your absence. Your colleague decided to check the health status of antelope populations by driving the main highway in Atlantis that runs along the outside of the island (see map below). Any antelope he and his team saw while transversing the highway they stopped and viewed for signs of morbidity or mortality. This is the data available to determine the spatial extent of the outbreak in Atlantian antelope.

What type of sampling design did your colleague use to determine the geographic extent of the outbreak?

With these data what can and can't you say about the extent of the outbreak in Atlantian antelope?

Question 4 (individual):

What agency(s), organization(s), university(s), or individual(s) may be your main point of contact for sample collection and diagnostic testing? Who may be able to you answer: what samples to collect; how to ship samples; what permits are needed; which laboratories have the appropriate resources?

Appendix A – The Dominion of Atlantis, General Information

(This country does not exist)

Cycle 4 Workshop - 2016

The Dominion of Atlantis is a parliamentary democracy with a capitalist economy.

1. Mostly self-sufficient in food production, 10% export balanced by 10% import
2. Main sources of wealth:
 - Agricultural products
 - * Major exports of poultry, cheese, farmed mink pelts and wine
 - * Sheep are particularly important. An ancient breed of sheep was brought to Atlantis by Vikings in the 8th Century BCE and has persisted on the Mallotus Islands since that time. They have a unique rich dark yellow fleece now of great commercial value and produce 4-6 lambs per ewe per year while grazing year round without supplemental feed. They also are world-renowned as a dairy breed for production of exquisite and unique sheep milk cheeses.
3. Forest products for export
4. Tourism
 - Wildlife viewing, seaside and forest natural environments, hunting and fishing
 - Important wildlife populations for tourism include
 - * Atlantian Antelope - 550
 - * White-tailed Deer - 30,000
 - * Moose - 2,000
 - * Bald Eagles - 800
 - * Black Bears - 3000
5. Commercial seafood harvest for export (finfish & shellfish)
6. Wind and tide-generated electricity
7. Banking (tax haven)

Location: An island in the North Hibernian Ocean (see map) Human Population:

1. 946,000 people
 - 30% rural
 - 70% in urban centres,
 - * 43% in the capital city of Bigtown
2. Wealth: Median Annual Family Income: US\$30,000 Size: 56,000 km² (130 km x 560 km)

Climate: North Temperate

1. Average summer temperature - +21°C
2. Average annual extreme temperatures: -10°C to +28°C
3. Annual precipitation: 1,500 mm, (300mm as snow in winter) Social Infrastructure:
4. Relevant National Ministries/Departments:
 - Ministry of Health
 - * Medical laboratory in Bigtown
 - * 16 Regional Hospitals
 - Ministry of Agriculture & Aquaculture
 - * Veterinary diagnostic lab in Epiville
 - * 10 Regional Offices
 - Ministry of Natural Resources (Fish and Wildlife Department)
 - * 18 Regional Offices
 - Ministry of Environment (Jurisdiction over Wilderness Areas and National Parks)
 - * 6 Regional Offices
 - Ministry of Ocean Resources (jurisdiction over ocean fish and marine mammals)
 - * 18 Regional Offices
 - Ministry of Tourism

Aboriginal Government

- Anguilla Original People's Council - Government for 20,000 aboriginal people which controls all resources on 5,000 km² of Atlantis, mostly adjacent to parks and wilderness areas. Special hunting and fishing rights extend to the whole country.

Universities:

- Harrison Lewis National University (20,000 students, Bigtown)
 - * Includes Atlantis Veterinary College
- 6 small (500 to 4000 students) regional universities distributed across country

Non-Government Organizations:

- National Farmers Association
- Atlantis Natural History Club (naturalists)
- National Fish and Game Association (recreational hunters and fishermen)
- National Fishermen's Union (commercial ocean fisheries)
- Golden Fleece Sheep Breeders Association

Calliope International (animal rights and welfare association)

Atlantian Antelope - Species Information - The Atlantian antelope (*Antilocapra atlantia*) is a species of antelope known to exist only on the Island of Atlantis. Males or bucks weigh 88 - 140 lbs (Adult), and females or does weigh 75 - 110 lbs (Adult). The most notable features of the males are a bright yellow cheek patch, ringed with red, and distinctive yellow markings adorning their horns. The females give birth to a single fawn every other year during the month of April. The Atlantian antelope is a diurnal species that spends most of the daylight hours foraging or loafing. During the night, the antelope bed in heavy cover. This species has a diverse diet, and forages on a wide variety of grass and browse species. This species is a herd animal with the herd comprising maternal groups. Yearling males are driven from the herd by the matriarch doe prior to parturition, and disperse throughout the island. Due to their limited geographic distribution, the Atlantian antelope is considered a highly endangered species, and it is included on the list of species protected by CITES. The Antelope are widely distributed throughout their range in Atlantis; however, they are found most abundantly in the provincial and national parks (light and green polygon on map). They inhabit a wide-range of habitat types on the Island from the coastal forests to the sage-brush steppes of central Atlantis.



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Each year the Ministry of Natural Resources conducts surveys throughout the Island using a grid system. They enumerate the number of antelope observed in each grid cell. The 2016 survey results are shown below. Recent counts have shown a growing populations. This population expansion has increasingly brought them in contact with domestic sheep, which are often free-ranging on the Island.

The Atlantian antelope is highly regarded among the Aboriginal peoples, and plays a prominent role in their folklore and religious beliefs. The antelope is also a symbol of national pride because Atlantis is the sole place it inhabits, and tourists from around the world come to view this species on its native range.

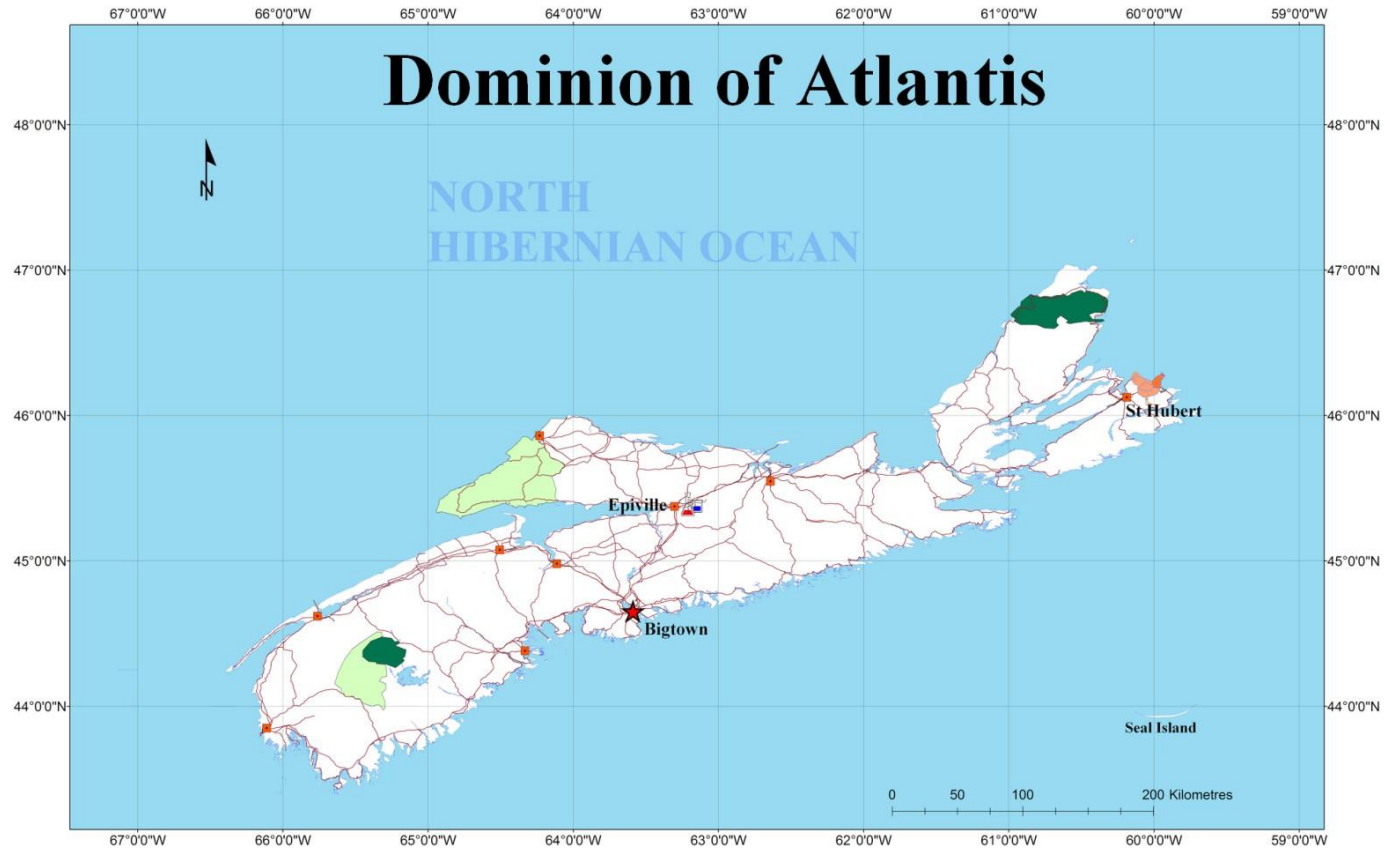


Figure 1: Map of the Island of Atlantis (light green -indicate provincial parks, dark green-indicate national parks).

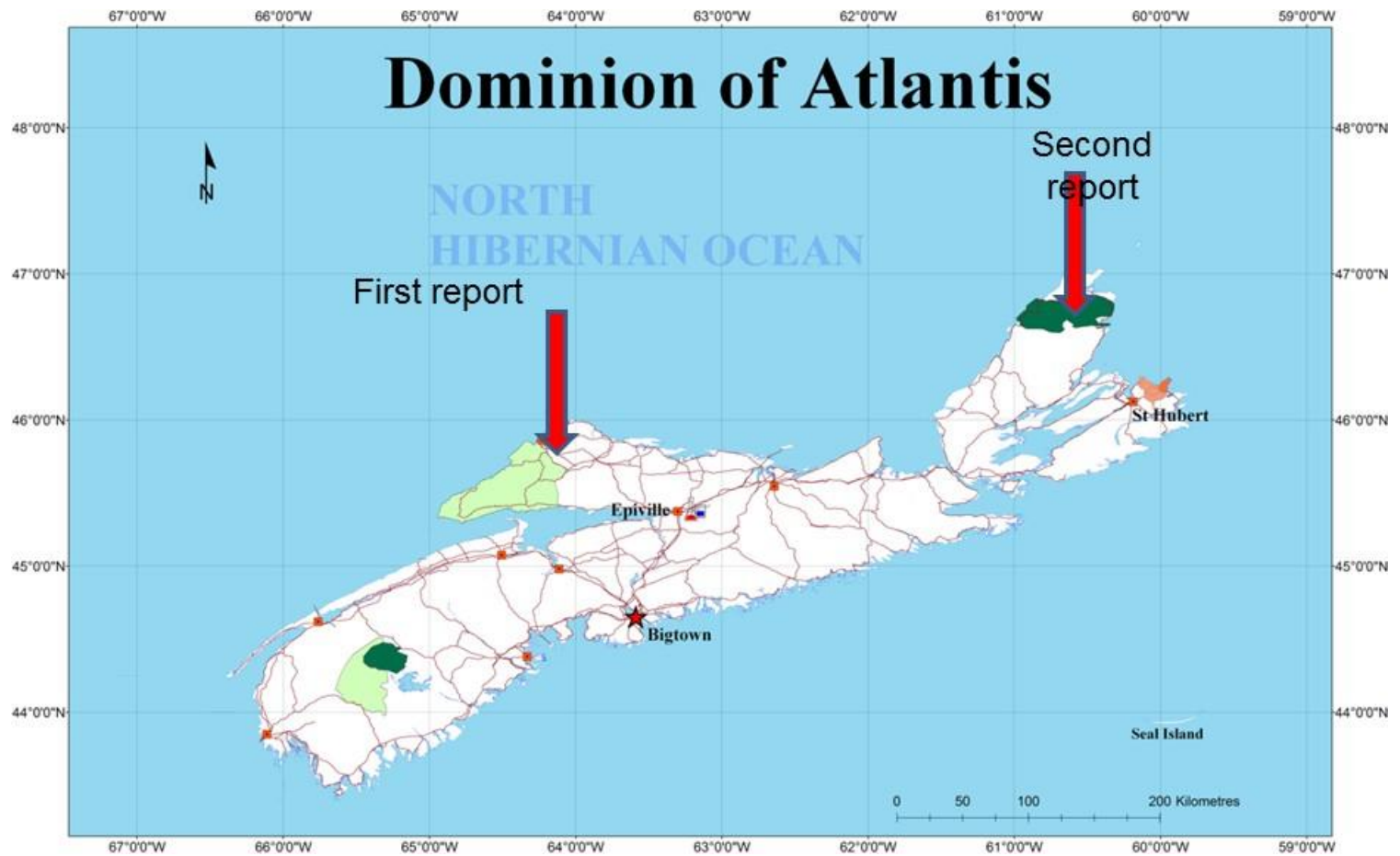


Figure 2: Location of the first reports of antelopes found dead.

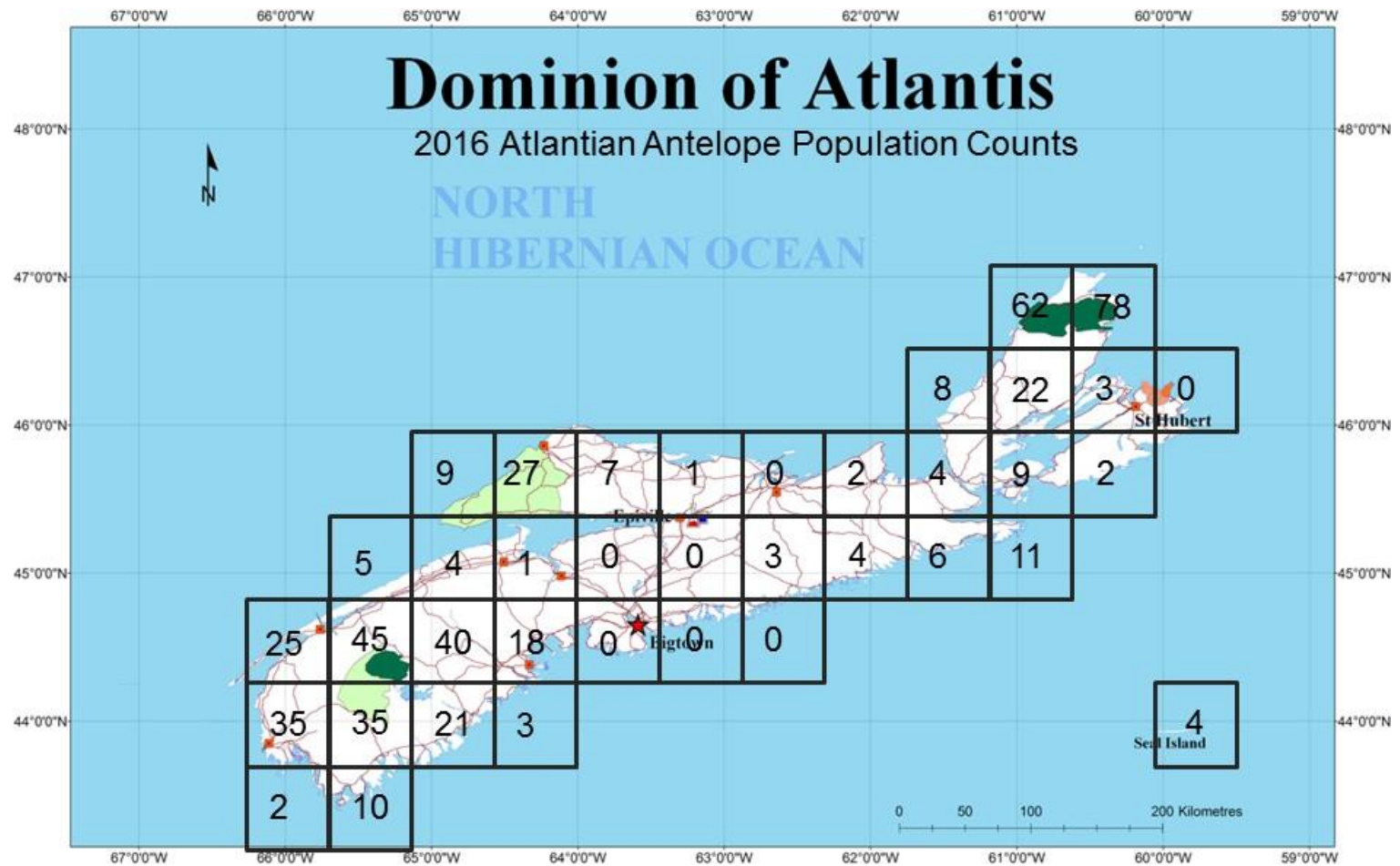


Figure 3: 2016 Atlantic survey information.

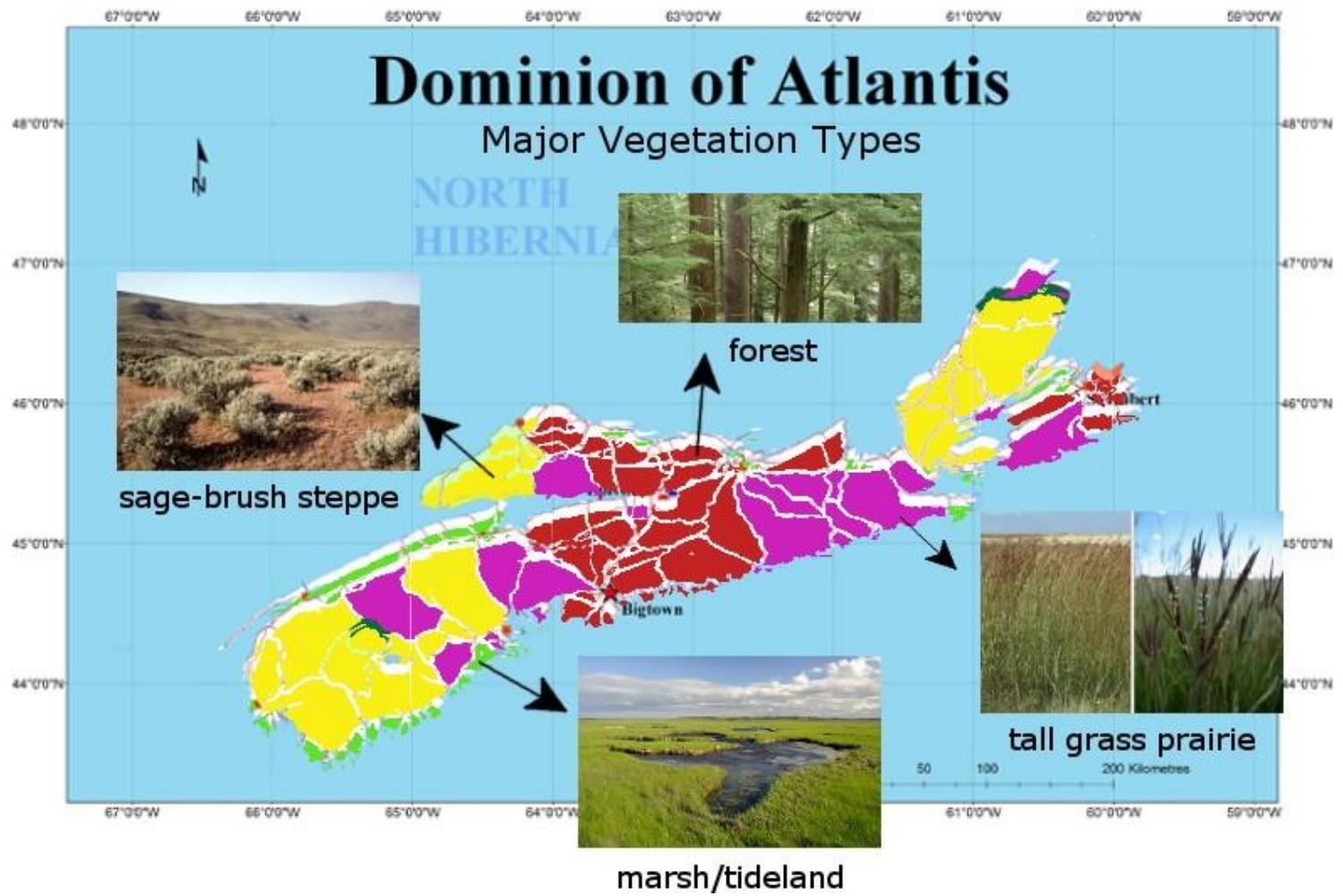


Figure 4: Vegetation map of Atlantis.

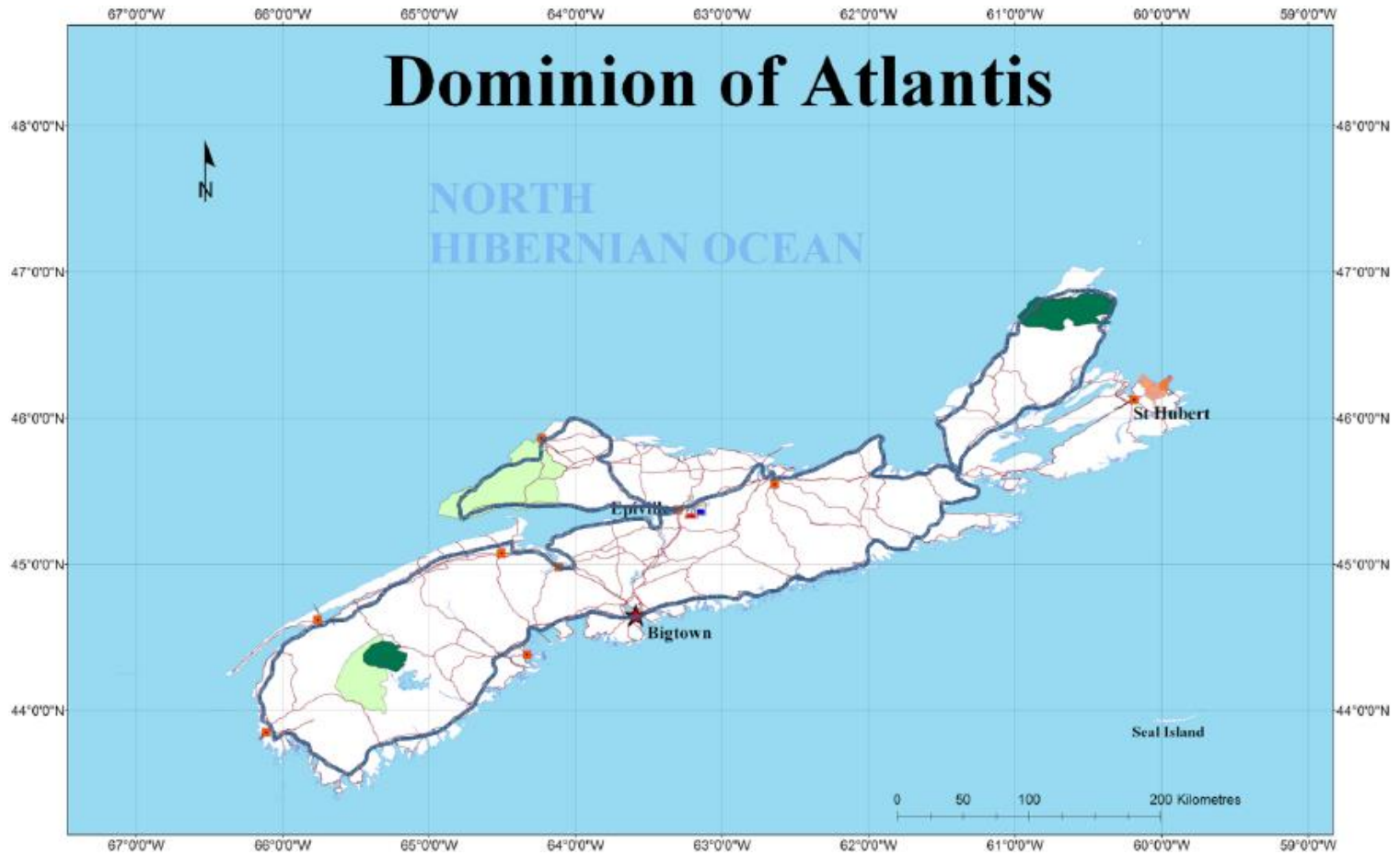


Figure 5: Map of survey route for assessing antelope population health.