ELISA for the Qualitative Determination of Canine Infectious Respiratory Diseases

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Canine Infectious Respiratory Diseases

Often referred to as “kennel cough”, canine infectious respiratory disease complex (CIRDC) is a series of bacterial or viral infections that result in the same presenting symptoms of coughing, sneezing, and nasal discharge to name a few. The classification of CIRDC encompasses a broader range of diseases including *Bordetella bronchiseptica*, *Chlamydophila*, *Mycoplasma spp.*, *Streptococcus equi subspecies zooepidemicus*, canine adenovirus type 2 (CAV-2), canine parainfluenza virus (CPiV), canine respiratory coronavirus (CRCoV), canine herpesvirus (CHV), canine distemper virus (CDV), canine influenza virus (CIV), canine reovirus, and canine pneumovirus.[1,2] Many of these pathogens are easily transmissible via droplet contact. Exposure may occur through nose to nose contact, vapor when coughing, saliva when sharing water bowls, or a canine licking another dog’s previously shed disease cells off of their paws. Rapid diagnosis is needed to prevent larger scale outbreaks.

Currently, the diagnostics for CIRDC are poor. Many of the diseases have the same presenting symptoms but require different treatments. To properly diagnose the illness, polymerase chain reaction (PCR) must be run in order to determine the genetic makeup of the virus.[3,4] PCR replicates the process of cell division. First, DNA is denatured in solution at 95°C. Such high temperatures break the hydrogen bonds in the double helix and force the two DNA strands to separate. Next, the solution is lowered to between 55°C and 65°C.[5] This change in temperature allows DNA primers to anneal to the now separated strands and DNA polymerase will become activated. DNA polymerase catalyses extension and replication is able to take place at approximately 72°C.[5] This process is repeated numerous times, resulting in multiple fold pieces of DNA being available for processing by gel electrophoresis.

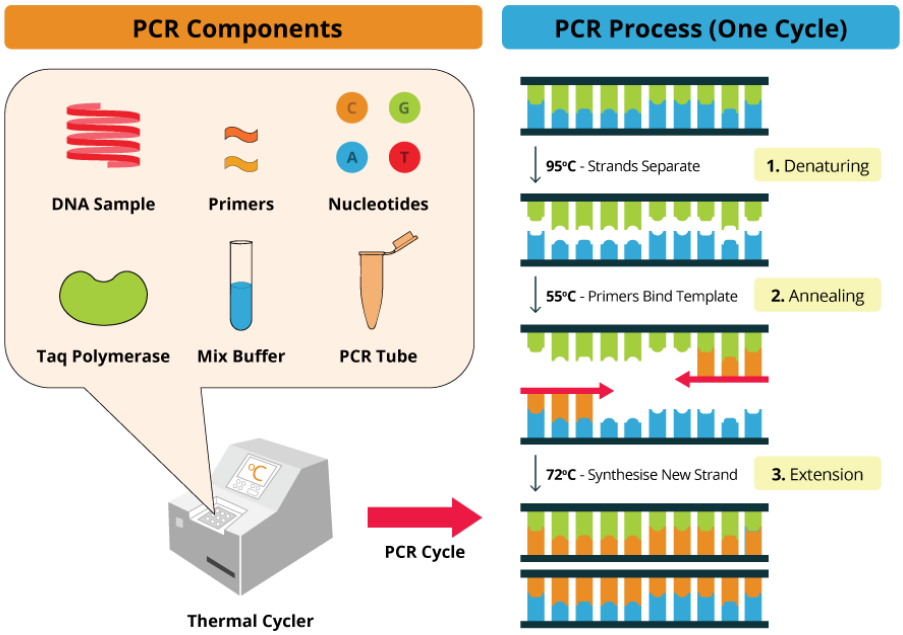

Figure 1. Schematic of Polymerase Chain Reaction (PCR). Image courtesy of Bosterbio.[5] Process is explained in depth above.

Gel electrophoresis separates digested DNA based on size of the molecule, with the smallest fragments moving the farthest. DNA has a partial negative charge, so it migrates toward the positive charge at the opposite end of the agar when an electric field is applied.

However, PCR can have a multitude of problems and is often not feasible. PCR may result in false negatives if the there are sample collection or handling issues, or sample collection does not fall within a very short window (usually less than 4 days) when the dog is most actively shedding diseased cells.[2,6] For example, influenza is shed early in the disease cycle and RNA degrades quickly when in storage. False positives can occur if the animal was vaccinated recently, the sample is contaminated or not handled properly, or poor lab technique is used.[2] For example, modified-live CDV vaccines have resulted in false positives for up to three weeks.[2,4] Other possible concerns include secondary or opportunistic diseases interfering with diagnosis, the disease

diagnosed is not actually the primary agent (or the source of the illness), or, in order to run a PCR, analysts must be using the correct primer for the intended disease.

Focusing on larger scale concerns, veterinary clinics are commonly small businesses that do not have a laboratory on-site. Even those that have an on-site lab often lack a thermocycler (the instrument used to perform PCR) due to cost. For example, one of the cheaper thermocyclers from MilliporeSigma costs $1,150.[7] Lacking a laboratory on-site means that biological samples must be sent to outside sources for analysis, a costly procedure. To run a full panel from Cornell University College of Veterinary Medicine, the baseline is $120.[8] While this panel covers most of the CIRDs listed above, the cost shown is only considering running the panel. It does not include the cost of an examination, sending the sample out, labor of those at the clinic, medications/treatments, or possible hospitalization of the animal. An examination for a respiratory concern can quickly become a costly veterinary bill that clients either don't want to pay or can't afford. In addition, PCR results are generally provided in 3-5 business days.[8] During this time, an animal is still actively shedding disease cells. Due to these complications, veterinarians must use what one practicing veterinarian referred to as a "shotgun" approach. The simple explanation is that doctors write prescriptions for antibiotics and tell clients to bring their animals back a week later if symptoms persist. There are several important drawbacks to haphazardly providing antibiotics:

1) Patients may develop antibiotic resistant diseases if the antibiotic provided is not sufficient for neutralizing that particular strain of illness.

2) Viruses cannot be treated with antibiotics and *Bordetella* (a bacteria) is unpredictably resistant to antibiotics.[3]

3) Not correctly diagnosing and treating an animal on the first visit results in more vet bills.

4) Not correctly diagnosing and treating an animal puts other animals at risk (including those at the clinic).

Thus, there are many reasons that rapid, affordable, and convenient methods for diagnosis of CIRDs are needed.

Parvo and How It Can Be Utilized

The methods of detection of canine parvovirus are potential models for the diagnosis of canine infectious respiratory diseases.

While parvo virus is capable of infecting many forms of life, including insects, dogs, cats, and humans, this paper focuses on Canine Parvovirus Type-2 (Parvo).[9] This disease affects the gastrointestinal tract of dogs. It's most common presenting symptoms are profuse vomiting, bloody diarrhea, and fever.[10,11] Death by parvovirus likely occurs within 48 to 72 hours after symptoms appear.[7] However, the disease itself has an incubation period of about three to seven days before clinical symptoms appear.[11] First discovered in 1976, it caused a worldwide epidemic by 1978. An effective vaccine was finally developed in 1979. Today's vaccine is a combination of solutions given as a single shot that combats parvo, distemper, canine adenovirus, and parainfluenza to name a few. Vaccines are given even 3 to 4 weeks when the puppy is between the ages of 6-16 weeks, with boosters recommended at 1 year of age, and every 3 years after. Due to genetic similarities, it is believed to have evolved from feline panleukopenia virus (FPV).[11]

Parvo acts by attacking the tonsils and lymph nodes in order to gain access to lymphocytes (white blood cells that they will then use to travel about the body). The immune response then begins killing lymphocytes, resulting in lymphopenia (and a major factor for infection by

secondary diseases). Lymphocytes are used to target the rapidly dividing cells in the gastrointestinal tract (particularly the crypts of Lieberkühn), the bone marrow, and the heart.[11]

One of the biggest reasons parvo is so deadly is dehydration. The primary cause of dehydration is through hemorrhagic diarrhea.[12] In the gastrointestinal tract, there are fingerlike projections called "villi" that increase the surface area of the gut to allow for better absorption of water and nutrients. Layering these villi are even smaller projections called "microvilli" that form a brush border.[13] In between the villi are cavities called crypts of Lieberkühn which are where rapid cell division takes place to form new cells to line the gastrointestinal tract. When parvovirus attacks these crypts, the lining of the gastrointestinal tract thins due to a lack of new cells.[12] Thinning of the lining between the bloodstream and gastrointestinal open-space (called the "lumen") results in blood in the stool. Since the villi are damaged and unable to absorb nutrients as efficiently, more osmolytes remain in the fecal waste. Osmolytes are compounds that control the flood of water across a gradient; these are substances such as sodium, potassium, chloride, and glucose.[14] This creates a hypertonic (or hyper concentrated) environment in the lumen and raises the osmotic pressure significantly. In order to restore homeostasis and return to an isotonic state, water is forced out of intestinal cells (following their electrochemical gradient).[14] Excess fluid in the stool causes diarrhea, which then leads to dehydration and death.

While years ago parvo was an almost guaranteed death sentence, advancements in treatment and detection have raised the survival rate to approximately 90%.[10] Due to its potential lethality, parvo has been highly investigated. Parvovirus can be diagnosed via a variety of testing methods including white blood cell count, a biopsy, PCR, antibody titers, and fecal enzyme-linked immunosorbent assay (ELISA).[15] The problem with parvo is that these tests are all qualitative.

However, research is currently being done to develop a quantitative test using charge detection mass spectrometry.[16]

Various Tests

White Blood Cell Count

White blood cell count is useful for determining the presence of parvo due to diseased cells attacking the bone marrow.[11] A low white blood cell count coupled with a positive ELISA test is a hallmark indicator of canine parvovirus. Due to a lack of ability to replenish blood cells as a result of damaged bone marrow, a blood transfusion is often recommended as a treatment for severe cases of parvo.

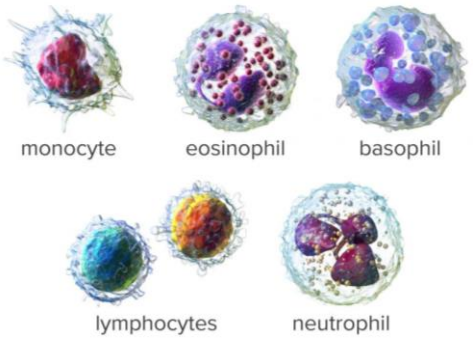

Figure 2. Examples of White Blood Cells. Image courtesy of Medical News Today.[17] Lymphocytes are the infection site of parvo.

Biopsy

The procedure for taking a biopsy is very similar to that for performing a white blood cell count. A trained medical professional will take a sample of tissue that is believed to be diseased and run a series of tests to help identify the pathogen. This often includes preparing a microscope slide by fixing the tissue on it.[18] One way to identify the cell type is by Gram-staining to determine whether a cell is gram-positive or gram-negative. Gram staining is performed by first using heat

to kill and fix the cells of interest to a slide. Next, crystal violet is added and allowed to absorb for 1 minute before being rinsed with deionized water. After that, iodine is added to the slide and allowed to absorb for 45 seconds before being rinsed with deionized water again. 95% Ethanol is then used to rinse the slide until just before color no longer drains from the sample. The slide is then rinsed with deionized water another time. Finally, safranin (a counterstain) is added and allowed to absorb for 1 minute before the slide is rinsed with deionized water and dried for viewing.[9] The importance of this procedure is that Gram-positive cells have a thicker layer of peptidoglycan which absorbs crystal violet and turns the cells on the slide visibly purple. Knowing the Gram-character of a microorganism is particularly important when treating pathogens. Gram-negative pathogens are generally more resistant to antibiotics due to double-membrane structure.[9]

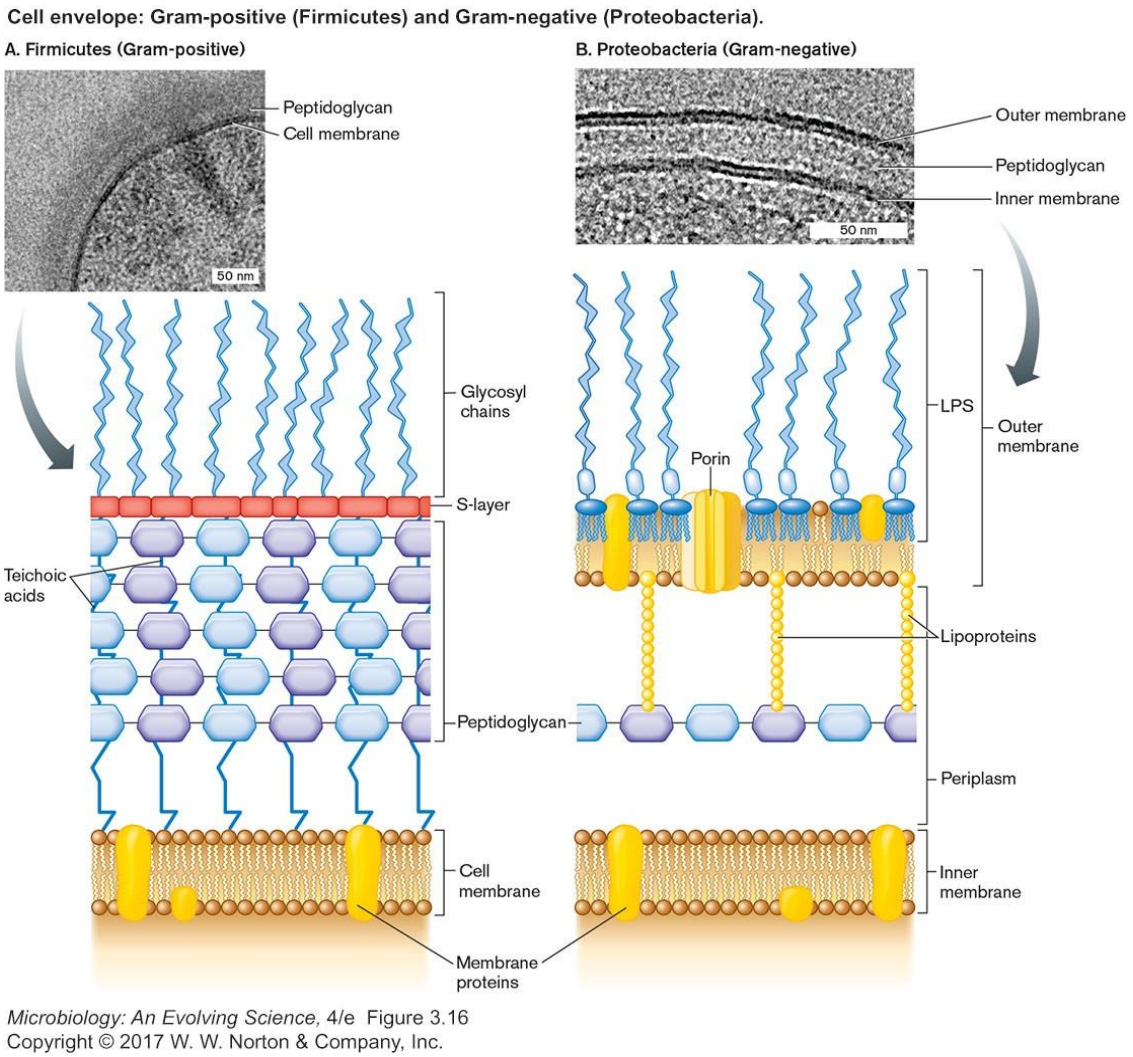

Figure 3. Comparison of Gram-positive and Gram-negative cells. Image courtesy of *Microbiology: An Evolving Science*. Gram-positivity is a character found in bacteria.[9] Gram-positive cells will turn purple when stained with crystal violet. Gram-negative cells will not.

If the cells are gram-negative, the crystal violet will not be retained and instead the cells will turn pink from the safranin. However, if rinsed with too much ethanol after the iodine stain, all of the crystal violet will rinse out and the cells will turn orange. Since parvo is gram-positive, the cells will appear purple when stained.

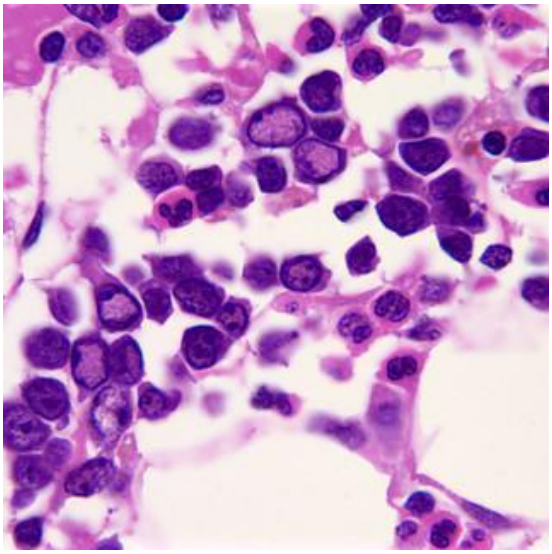

Figure 4. Canine Parvovirus. Image courtesy of David Grier.[19] Gram-stained parvovirus in the bone marrow. Note the purple color and coccus (spherical) character.

Titers

The growth of prevalence of ELISA testing has led to the titers test being relatively obsolete. A titer tests for the level of antibody present in an animal (including humans).[15] This test is performed by taking a blood sample and can be used to determine the strength of an immune response and whether an individual is immune to a pathogen or currently fighting infection. These results also allow professionals to discern if a patient is building antibodies against the vaccine itself rather than the pathogen (another important reason to properly diagnose your pets).[20]

There are five types of titers tests: IgA, IgD, IgE, IgG, and IgM.[21] Ig is short for immunoglobulin while the letter is the subclass of antibody depending on its location and function in the body. Immunoglobulin A is commonly found in mucosal secretions, specifically in the digestive and respiratory tracts. Immunoglobulin D is commonly found in the bloodstream, but its mechanism is not well understood. Immunoglobulin E is commonly found in the lungs, skin, and mucous and is most active during allergic reactions. Immunoglobulin G is found in all bodily fluids

and is most closely associated with fighting bacterial and viral infections. Immunoglobulin M is found in the blood and lymph and is the first antibody created at the onset of infection.[21]

The two primary types of titer tests are for IgG and IgM.[15] IgG titers are a measure of long-term antibodies produced. IgG titers are not reliable in older dogs as results just indicate the vaccines as puppies were effective whereas high values in puppies without a vaccination history are more indicative of infection. IgM titers test for recent antibody production.[15] High values here are likely to be more conclusive of the presence of infection. Due to the ability of vaccinations to interfere with the outcomes of titers tests and the difficulty many doctors have interpreting their results, ELISAs are the preferred method of diagnosis.

Figure 5. Temporary Image That I Found Funny. Image courtesy of Bonfire.[22] Just for giggles because I couldn't find a picture of the test.

ELISAs

When a foreign body enters ones' anatomy, the immune response is activated. The immune response has two portions: innate and adaptive. The innate immune response is immediately triggered by the recognition of an unfamiliar object in the body. The adaptive immune response

recognizes antigens on the surface of invading cells and releases antibodies that will bind to those specific antigens at the epitopes (or binding sites) in order to neutralize them.[9,23] Antibodies are Y-shaped proteins composed of 4 subunits: 2 light chains and 2 heavy chains held together by disulfide bridges. The V shaped component of the antibody is known as the variable region. Within the variable region is the "fragment antigen-binding" or Fab region and is what makes antibodies so specialized to their antigens. The tail of the Y is the Fc region and is specific to the species you are working with. This means that a dog's Fc region is different from that of a mouse but the same as that of another dog. It is for that reason that ELISAs work.[23,24]

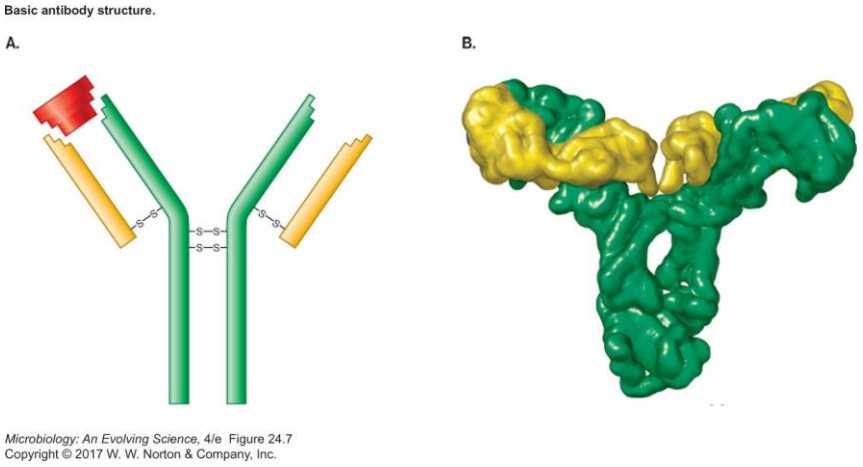

Figure 6. Basic Structure of an Antibody. Image courtesy of *Microbiology: An Evolving Science*.[9] Two models that allow for visualization of the heavy (green) and light (yellow) chains.

ELISA is the most common clinical diagnostic test in practice today. Antibodies are passively bound to a polystyrene surface. A protein that will not bind to the antibody (usually bovine serum albumin) is then washed over the surface to cover areas not already bound in order to prevent nonspecific binding of reagents. The sample is then added to the ELISA and incubated for a period of time to allow the analyte to bind to the assay. ELISAs often provide a colorimetric result due to the enzyme-conjugated antibodies present. While ELISAs can be used to crudely

provide quantitative results (such as with a dipstick in urine or pH strips) they are more widely used qualitatively (i.e. a pregnancy test will either provide a positive or negative result).[25]

Charge Detection Mass Spectrometry

Charge detection mass spectrometry (CDMS) can be used to determine the binding affinity of antibodies with their antigens. CDMS simultaneously measures the mass-to-charge ratio (m/z) and charge in individual ions.[26] This method is useful because there is not an upper limit to the mass that can be measured and it can be used on complex mixtures. CDMS is unique in its approach to traditional mass spectroscopy because, instead of yielding m/z, this method allows the analyst to determine the exact mass of a compound. The application here is that scientists can determine the mass of a canine parvovirus (CPV) capsid saturated with Fabs and subtract the mass of an unbound CPV capsid to get the difference (or the mass of bound Fabs). Determining the mass of bound Fabs is a novel approach for engineering antibodies with increased binding affinity for specific pathogens.

CDMS works by changing pressure to 'trap' molecules in specific conformations.[26] This trapping is particularly helpful in the case of viruses and other pathogens. In the process of replicating, viruses experience conformational changes. Conformational changes are necessary in order for the virus to inject their genetic material into a host cell. CDMS is able to detect the change from a late stage intermediate to a bound conformation of trapped molecules.[27]

One of the goals of CDMS is using conformational changes to determine the stoichiometry of antibody binding to viral particles. Current methods for measuring the stoichiometric equilibrium between proteins and ligands include isothermal titration calorimetry (ITC), surface plasmon resonance (SPR), ELISA, and Forster resonance energy transfer (FRET). ITC measures

the environmental parameters that result in peak binding affinity (such as pH and temperature).[28] SPR detects the oscillation of electrons' ability to store electrical energy when interacting with light (permittivity). Permittivity can either be positive or negative.[29] FRET uses a fluorophore to transfer fluorescence from one molecule to another in close range.[30] However, these methods provide little stoichiometric information as they cannot detect binding to individual viral particles.

CPV is an ssDNA virus with a T=1 icosahedral capsid (meaning that the capsid is composed of 60 asymmetrical units on one protein).[31,32] Dunbar et. al. (2018) studied four viral antibodies (chosen using cryogenic electron microscopy) binding to CPV using CDMS in order to measure binding affinity and determine a stoichiometric relationship between CPV capsids and antibodies.[16] They discovered that each antibody had a unique CDMS profile. Masses of each Fab were preliminarily identified with liquid chromatography-mass spectrometry (LC-MS), the conventional method.[16] CPV capsids containing DNA and those that are empty after insertion into the cell looked identical under X-ray crystallography, despite CPV capsids having three distinct surface proteins.[16] However, CDMS definitely showed that empty capsules have smaller masses. Adding increasing concentrations of Fabs resulted in increased mass of the compound when analyzed using CDMS (Fig. 7).[16] When comparisons were made between Fabs E, B, 14, and B using binding curves, Dunbar et. al. were able to conclude that different antibodies for the same pathogen have different binding affinities and that concentration impacted binding affinity (following the Le Chatelier's Principle and the Law of Mass Action) (Figures 8-10). Also, CPV capsid degradation resulted in lower binding affinity (Fig 11).

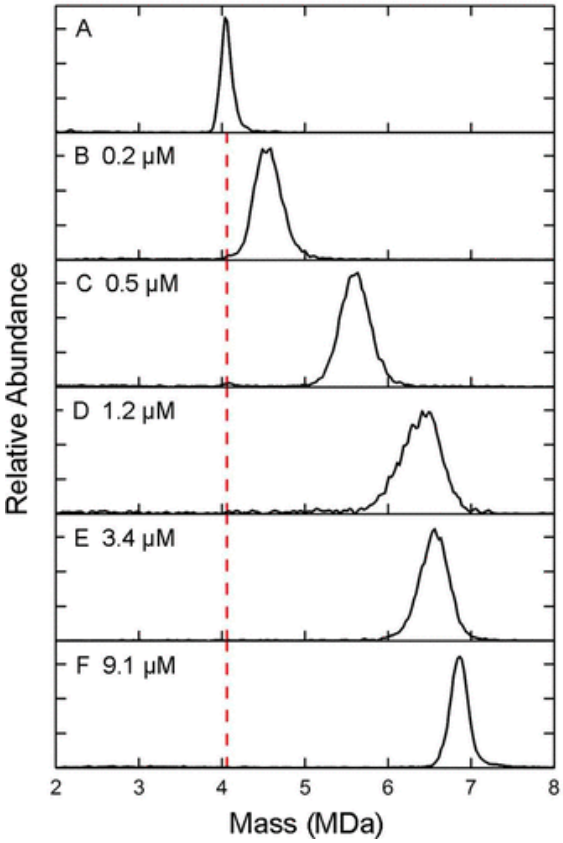

Figure 7. Increasing Fab Concentration Increases Mass. Image courtesy of Dunbar et. al. CDMS mass histograms measured for the reaction between CPV and Fab E. (A) Mass histogram of empty CPV with no Fab. The center of the mass distribution of unreacted CPV is indicated by the red dotted line at 4.06 MDa. B–F) are the CDMS histograms of CPV reacted with increasing concentration of Fab E: (B) 0.2 μM, (C) 0.5 μM, (D) 1.2 μM, (E) 3.4 μM, and (F) 9.1 μM.[16]

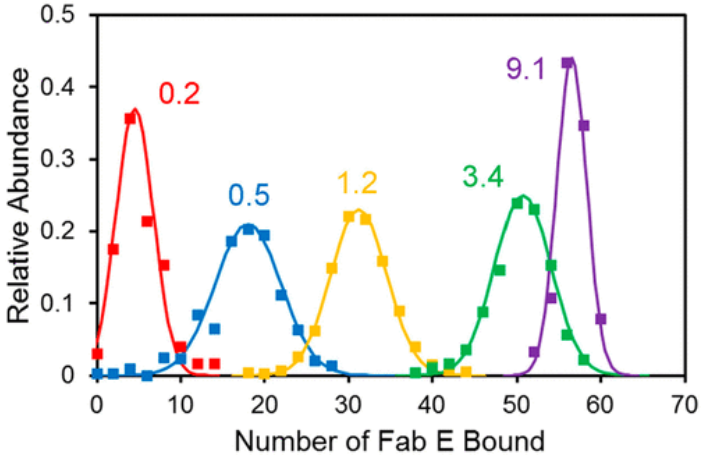

Figure 8. Number of Fabs Bound Affects Binding Affinity. Image courtesy of Dunbar et. al. Graph comparing the concentration of Fabs present to the relative abundance (a measure of binding affinity).[16]

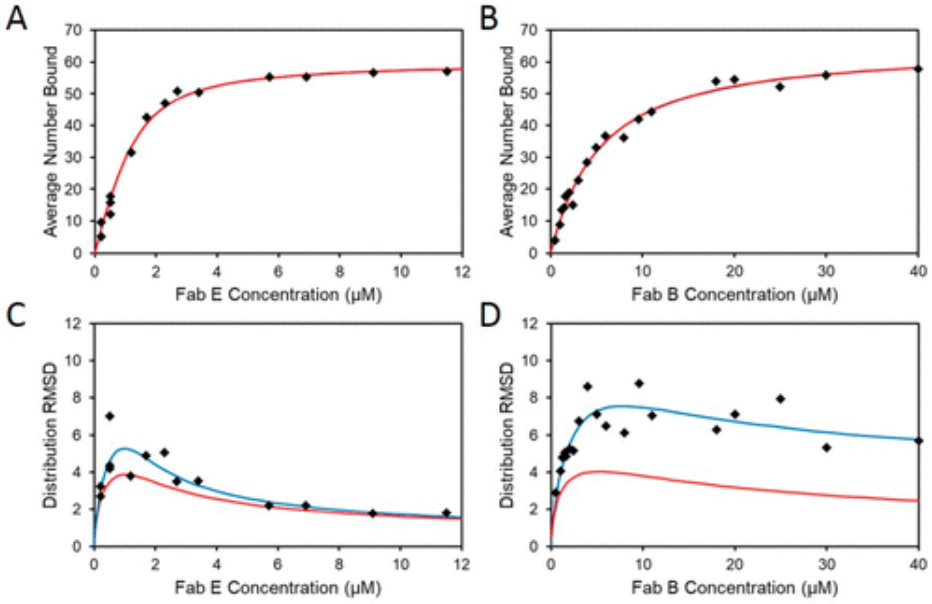

Figure 9. Binding Curves and Root-Mean-Square Distributions (RMSDs) for Fabs B and E. Image courtesy of Dunbar et. al. The binding curves are plots of the average number of Fabs bound versus initial Fab concentration. The points are the observed values. The red lines show the prediction of the standard model for ligand binding (assuming all sites have the same affinity). The blue lines show the predictions of a model where CPV capsid subpopulations have different intrinsic affinities. In the binding curve plots, the blue line is hidden by the red.[16]

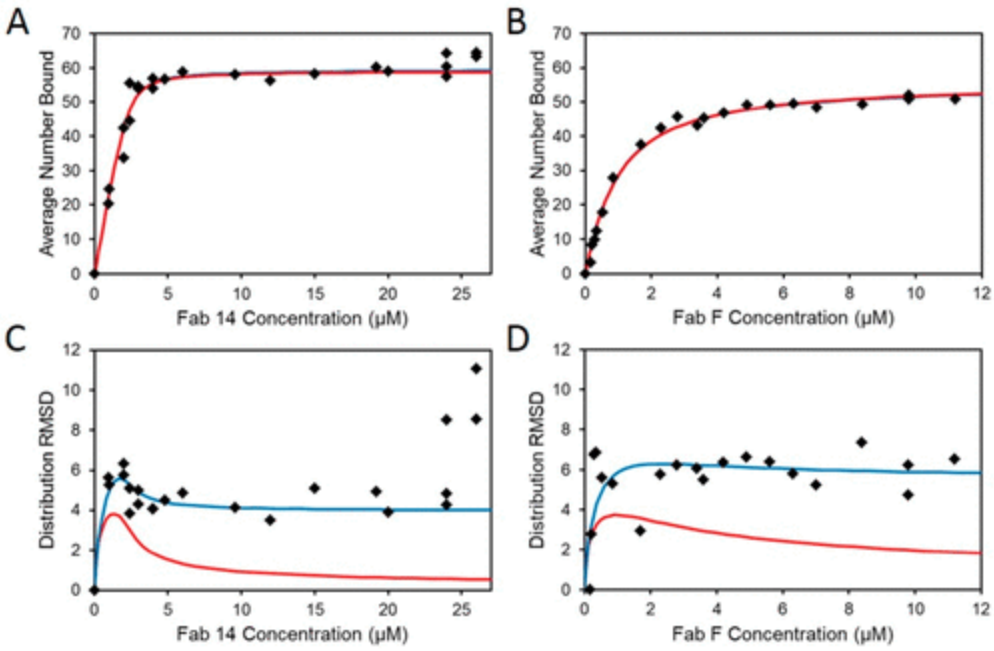

Figure 10. Binding Curves and Root-Mean-Square Distributions (RMSDs) for Fabs 14 and F. Image courtesy of Dunbar et. al. The binding curves are plots of the average number of Fabs bound versus initial Fab concentration. The points are the observed values. The red lines show the prediction of the standard model for ligand binding (assuming all sites have the same affinity). The blue lines show the predictions of a model where CPV capsid subpopulations have different intrinsic affinities. In the binding curve plots, the blue line is hidden by the red.[16]

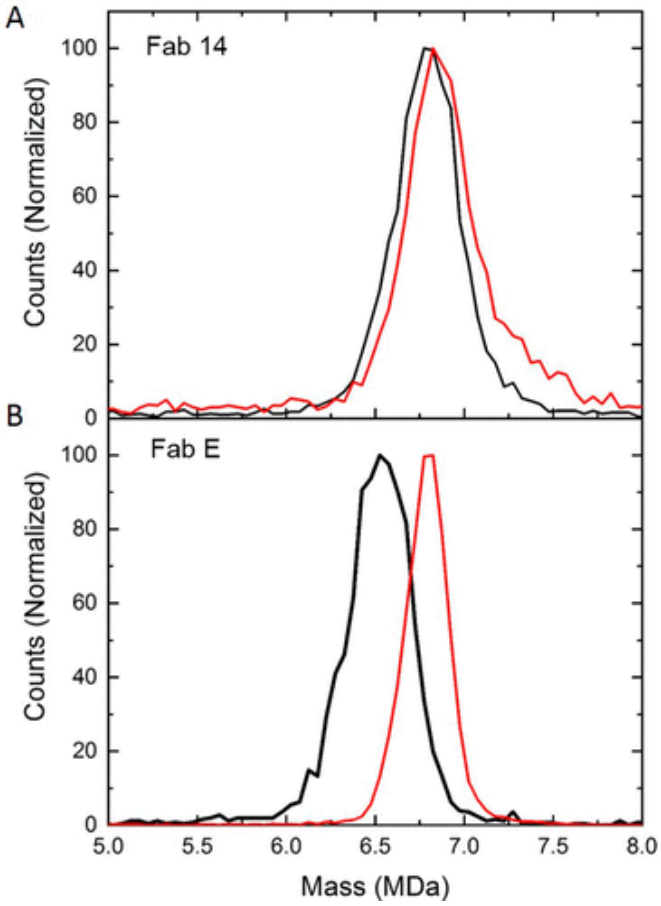

Figure 11. Demonstration of Capsid Degradation in Relation to Binding Affinity. Graph comparing mass of new and aged CPV capsids when combined with Fab 14 (19.2 μM) and Fab E (5.7 μM). The black line shows the distributions measured for aged capsids, and the red line shows the results for new capids. Lower mass is a consequence of lower binding affinity.

There are distinct changes in mass between CPV capsids, CPV capsids bound to (but not saturated with) Fabs, and CPV capsids saturated with Fabs. Researchers were able to determine that different subspecies of parvo virus had different binding affinities to the Fabs they experimented with. Determining binding affinities for various Fabs in multiple strains of a disease will allow for more accurate detection and therefore more precise treatment of pathogens.

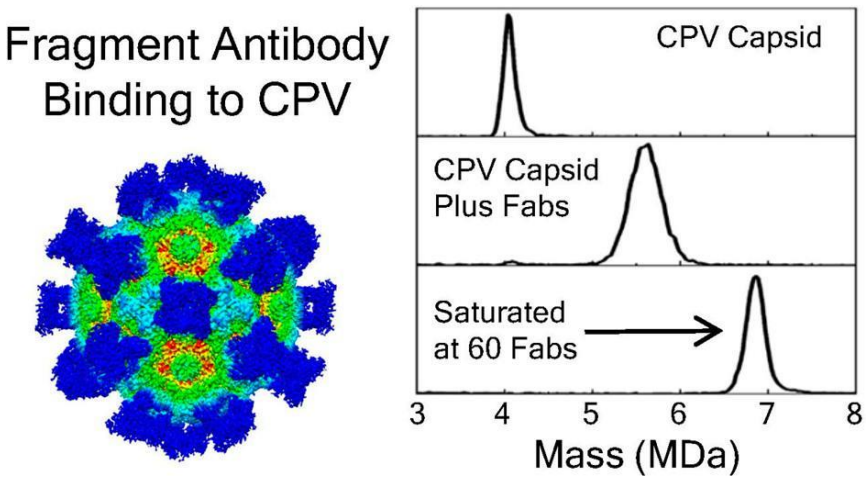

Figure 12. Mass Spectrum of Canine Parvovirus. Image courtesy of Dunbar et. al.[16] Comparison of mass spectral analysis of CPV capsid when unbound to that of one bound with some antibodies and one completely saturated with antibodies.

Next Steps

The next step for rapid, affordable, and convenient detection of CIRDs is to create a handheld ELISA test with multiple assays on it. For *VetScan* Canine Parvovirus Rapid Test from Abaxis,[33] a veterinary professional takes a rectal swab of the animal and inserts the swab back into the holding chamber. A vial at the top of the chamber is then rocked back and forth several times in order to crack the blue seal and release the extraction buffer solution into the holding chamber to be mixed with the sample. The vial is then squeezed 5-6 times in order to mix the solution and emulsify the cells. Finally, 3 drops (approximately 150 µL) of the fecal solution is transferred to a designated portal on the test strip and allowed to incubate for several minutes before results are read. While this test is primarily intended for qualitative results, a moderately quantitative result can be deduced as strong positive tests will provide results in as little as 1 minute. However, most results are produced in 10 minutes, and results appearing after 15 minutes should be disregarded.[30] The sensitivity and specificity were both described to be about 96.9% due to the correct

identification of 93/96 hemagglutination inhibition (HI) positive canine fecal samples and 157/163 HI negative canine fecal samples.[33] HI assays are used to measure the virus' ability to bind (hemagglutinate) to red blood cells and prevent clotting.[34]

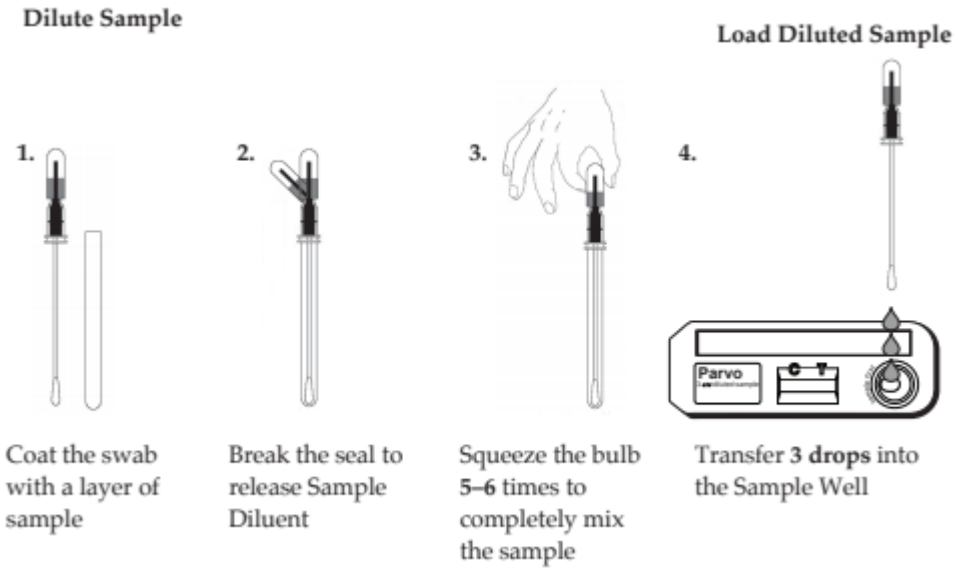

Figure 13. Parvovirus Test Procedure. Image courtesy of Abaxis.[33] Diagram briefly demonstrating how a *VetScan* Canine Parvovirus Rapid Test is used.

Extraction buffers are designed to dissolve cell walls in order to access the virus hiding inside the lymphocytes, as well as dilute the concentration of microbes. The exact chemical makeup of the *VetScan* Rapid Test extraction buffer is not disclosed by Abaxis (per a materials specialist), but the instructions packet states that approximately 0.2% of this particular solution is composed of sodium azide.[33] Sodium azide is instrumental in halting cellular respiration and aerobic metabolism by preventing the cell from using oxygen.[35] Azide has a mechanism that functions similarly to that of cyanide. These compounds bind to the A-3 heme portion of cytochrome C oxidase in Complex IV, preventing the binding of oxygen as a terminal electron

acceptor.[36] This disrupts the proton concentration gradient that is necessary to rotate complex V in order to generate the high-energy molecule adenosine triphosphate (ATP).

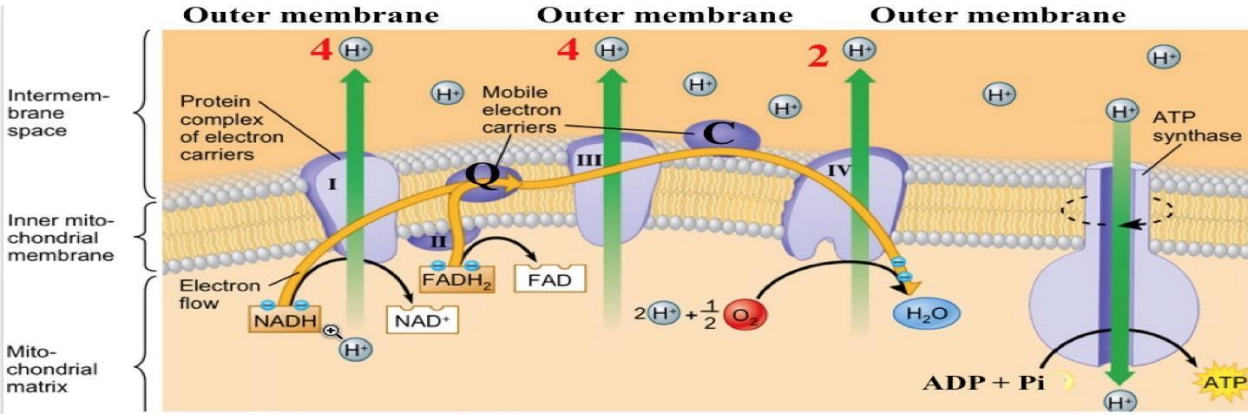

Figure 14. Electron Transport Chain. Image courtesy of the University of New Mexico.[37] The proton gradient formed by the electron transport chain is necessary for the aerobic production of ATP.

Because there are vaccines for several viral forms of CIRDC, we know that medical professionals have an approximate idea of the Fab regions of these diseases. A similar style of test could be utilized for CIRDs by substituting a rectal swab for a nasal swab. However, adjustments would need to be made to the extraction buffer, as the buffer currently in use does not dissolve mucus.[33] Further testing would be needed to determine the appropriate reagents to liquidate the mucus in the nasal samples without digesting the microbes' genetic material. The use of CDMS to determine the binding affinity of several strains of each disease would allow for the ELISAs to become more specific and test for multiple strains of a single virus. Once a positive result is confirmed with a broad CIRD ELISA, a second ELISA could be run with multiple different strains of the indicated virus. By these methods, veterinarians would have the ability to diagnose CIRDCs

like never before. These advancements would allow medical professions to treat animals more on a case-by-case basis with minimal cost to the owner as well as allow staff to determine the best protocol for preventing or containing an outbreak.

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References

University of Wisconsin-Madison School of Veterinary Medicine. Canine Infectious Respiratory Disease Complex (CIRDC, a.k.a. "Kennel Cough"). https://www.uwsheltermedicine.com/ library/resources/canine-infectious- respiratory-disease-complex-a-k-a-kennel-cough (accessed on Dec 13, 2019).

Gober, M.; McCloskey, R. Canine Infectious Respiratory Disease (CIRD). *Zoetis Technical Bulletin*, October 2017, pp 1-16.

Keil, D.J.; Fenwick, B. Canine Respiratory Bordetellosis: Keeping up with an Evolving Pathogen. *Recent Advances in Canine Infectious Diseases*, January 2000, pp 1-6.

Pantchev, A.; Sting, R.; Bauerfeind, R.; Tyczka, J.; Sachse, K. The detection of Chlamydophila psittaci genotype C infection in dogs. *Vet. J.* **2009**, *181*(3), 274-279.

(5) Bosterbio. Polymerase Chain Reaction (PCR) Key Principles. https://www.bosterbio.com/ protocol-and-troubleshooting/molecular-biology-principle-pcr (accessed on Feb 24, 2020).

Crawford, C. Canine Respiratory Infections in Animal Shelters. *Maddie's Shelter Medicine Program College of Veterinary Medicine*, 2011, pp 1-6.

MilliporeSigma. Thermocycler. https://www.sigmaaldrich.com/catalog/search?term=thermocycler&interface=All&N=0&mode=match%20partialmax&lang=en&region=US&focus=product (accessed on Dec 13, 2019).

Cornell University College of Veterinary Medicine. PCR Panels for Canine and Equine Respiratory Disease. https://www.vet.cornell.edu/animal-health-diagnostic-center/news/pcr-panels-canine-and-equine (accessed on Dec 13, 2019).

Slonczewski, J.; Foster, J.W.; Gillen, K.W. *Microbiology: An Evolving Science*, 4th ed.; W. W. Norton & Company, New York, 2017; all pages.

(10) American Veterinary Medical Association. Canine parvovirus. https://www.avma.org/resources-tools/pet-owners/petcare/canine-parvovirus (accessed on Dec 13, 2019).

Baker Institute for Animal Health. Canine Parvovirus. https://www.vet.cornell.edu/departments-centers-and-institutes/baker-institute/our-research/animal-health-articles-and-helpful-links/canine-parvovirus (accessed Feb 24, 2020).

Nandi, S.; Kumar, M. Canine Parvovirus: Current Perspective. *Indian J. Virol.* **2010**, *21*(1), 31-44.

Mueller, C.J. Principles of Animal Nutrition, 2020.

Cain, S.D. Cell Structure and Function, 2020.

(15) Mar Vista Animal Medical Center. Diagnosis of Canine Parvovirus. https://marvistavet.com/ diagnosis-of-canine-parvovirus.pml (accessed Dec 13, 2020).

Dunbar, C.A.; Callaway, H.M.; Parrish, C.R.; Jarrold, M.F. Probing Antibody Binding to Canine Parvovirus with Charge Detection Mass Spectrometry. *J. Am. Chem. Soc.* **2018**, *140*(46), 15701-15711.

Nall, R. What to know about white blood cells. https://www.medicalnewstoday.com/ articles/327446 (accessed on Feb 25, 2020).

National Cancer Institute. Definition of biopsy. https://www.cancer.gov/publications/dictionaries/ cancer-terms/def/biopsy (accessed on Feb 25, 2020).

Grier, D. Parvovirus inclusions in bone marrow. https://www.flickr.com/photos/david_grier/ 218395391 (accessed on Feb 25, 2020).

(20) Krause, L. Antibody Titer Test. https://www.healthline.com/health/antibody-titer (accessed on Feb 25, 2020).

Nemours. Blood Test: Immunoglobulins (IgA, IgG, IgM). https://kidshealth.org/en/parents/test-immunoglobulins.html (accessed on Mar 20, 2020).

Bonfire. Show Me Your Titers. https://www.bonfire.com/show-me-your-titers-1/ (accessed on Feb 25, 2020).

Nelson, C.D.S.; Palermo, L.S.; Hafenstein, S.L.; Parrish, C.R. Different mechanisms of antibody-mediated neutralization of parvoviruses revealed using the Fab fragments of monoclonal antibodies. *Virology,* **2007**, *361*(2), 283-293.

Cain, S.D. Antibodies. 2019.

(25) He, J. *The Immunoassay Handbook: Theory and Applications of Ligand Binding, ELISA and Related Techniques*, 4th ed.; Elsevier Science: U.S.A. 2003; pp. 381-394.

Keifer, D.Z.; Shinholt, D.L.; Jarrold, M.F. Charge Detection Mass Spectrometry with Almost Perfect Charge Accuracy. *Anal. Chem.* **2015**, *87*(20), 10330-10337.

Pierson, E.E.; Keifer, D.Z.; Selzer, L.; Lee, L.S.; Contino, N.C.; Wang, J.C.Y.; Zlotnick, A.; Jarrold, M.F. Detection of Late Intermediates in Virus Capsid Assembly by Charge Detection Mass Spectrometry. *J. Am. Chem. Soc.* **2014**, *136*(9), 3536-3541.

Duff, M.R.; Grubbs, J.; Howell, E.E. Isothermal Titration Calorimetry for Measuring Macromolecule-Ligand Affinity. *J. Vis. Exp.* **2011**, *55*, 2796.

Tang, Y.; Zeng, X.; Liang, J. Surface Plasmon Resonance: An Introduction to a Surface Spectroscopy Technique. *J. Chem. Educ.* **2010**, *87*(7), 742-746.

(30) Ben-Johny, M.; Yue, D.N.; Yue, D.T. Detecting stoichiometry of macromolecular complexes in live cells using FRET. *Nat. Commun.* **2016**, *7*, 13709.

Cotmore, S.F.; Tattersall, P. Parvoviruses: Small Does Not Mean Simple. *Annu. Rev. Virol.* **2014**, *1*, 517-537.

Swiss Institute of Bioinformatics. T=1 icosahedral capsid protein. https://viralzone.expasy.org/1057?outline=all_by_protein (accessed on Mar 20, 2020).

Abaxis. Canine Parvovirus Antigen Test Kit, Blot Test. https://www.abaxis.com/sites/default/files/resource-packages/Canine%20Parvo%20Rapid%20Test%20PI_0.pdf (accessed on Feb 25, 2020).

Bertran, K.; Susta, L.; Miller, P.J. *Egg Innovations and Strategies for Improvement*, Elsevier Science: U.S.A., 2017; pp. 547-559.

(35) Centers for Disease Control and Prevention. Sodium Azide Poisoning. https://emergency.cdc.gov/agent/sodiumazide/tsd.asp (accessed on Mar 20, 2020).

Johns Hopkins Bloomberg School of Public Health. Cyanide. http://www.centerforhealthsecurity.org/resources/fact-sheets/pdfs/cyanide.pdf (accessed on Mar 20, 2020).

University of New Mexico. ETC Story. https://www.unm.edu/~lkravitz/Exercise%20Phys/ETCstory.html (accessed on Mar 20, 2020).