

The Future of Biodetection Systems: Sampling

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Introduction

- Tetracore, Inc. Rockville, MD
- Immunodiagnostic and PCR based tests for infectious agents
- Government contracting
- Commercial assay development and production
 - Veterinary diseases (USDA licensed manufacturing)
 - Biothreat agents (FDA certified manufacturing)



Introduction

- The analytical process can be broken down into three stages: Specimen acquisition, processing and analysis
- There is enormous variation in the types of materials that must be sampled and this drives acquisition and processing methods
- Most systems feed into a relatively limited number of commonly used processing techniques. This is especially true for molecular analysis.



Sampling and Processing: From What

- Environmental
 - Various forensic matrices
 - Air filters and collectors
 - Water and soil
 - Feed lot runoff
 - Soil biomass
 - Fecal contamination of water
 - Food
 - Meat, produce, dairy, juices
- One common characteristic of many of these sample types is a small amount of target in a vast amount of sample.
- Culture is very effective but slow



Sampling and Processing: Clinical

- Human and veterinary
 - Blood, plasma, sera etc.
 - Stool
 - Infectious disease diagnostics
 - *Helicobacter pylori*
 - Norovirus
 - Cancer diagnostics
 - Pathogen control in livestock
 - *Campylobacter* in chickens (cloacal swabs)
 - Johne's (cow manure)

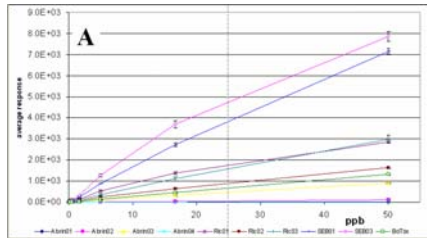


Sampling and Processing: For What

- Immunodetection
 - Antigen capture integral to most techniques
 - Plates, beads, lateral flow assays or arrays
 - Lectins occasionally used for capture
- Mass Spectrometry
 - Many of the same capture procedures used
- Molecular Identification
 - Nucleic acids bind to silica



Immunodiagnosics



Luminex multiplex detection of abrin, ricin, SEB and botulinum toxin in beverages. Samples were diluted in 100 μ L of fruit juice, dairy beverage, chocolate milk and soda.

- One advantage of an antigen capture immunodiagnostic test is that the capture antibody can act to concentrate the target
- Effectiveness of this is better on bead based systems when compared to planar surfaces
- Much of the work in this area applied more to detection technologies
- Capture of target on paramagnetic beads for eventual molecular analysis has been used



Processing for Molecular Analysis

- Collection of Specimen
- Concentration of Target – (Depending on requirements)
 - If dilute, concentration of target prior to extraction may be advantageous
 - Both specific and nonspecific means have been employed
- Extraction/Lysis (Not always necessary)
 - Usually leads to further concentration of the nucleic acids
 - Usually removes PCR inhibitors



Molecular Analysis: Extraction

- Effectiveness of extraction protocols can be marginal, especially considering time and expense
- Concentration is on the order of ten to twenty fold in most commercial kits assuming 100% recovery
- Much lower efficiency not unusual



Nucleic Acid Purification

- Most common method is based on the binding of DNA to glass under high salt concentrations
- Sample rinsed several times and eluted under low salt
- Commercial methods use glass impregnated membranes, filters or glass beads
- Fluids moved by centrifugation or vacuum.
- Beads frequently used in manual or robotic systems and depend on magnetic capture of the beads



Example of Processing Aerosol Samples

- A column purification protocol is described by OSHA for Dry Filter Units
- The unit collects on a 47mm polyester filter
- Reported detection limits are 2 CFU per filter by culture and 200 CFU per filter by PCR



Recovery From Dry Filter Units (DFU)

- Protocol described by OSHA
 - 30-40mL buffer, shake 30 minutes, transfer supernatant to new tube
 - Allow to settle 5 to 10 minutes and transfer supernatant
 - Centrifuge 30 minutes
 - Resuspend pellet in 1mL
 - Transfer 100µL to 100µL of media. Allow to germinate.
 - Perform a DNA extraction on a spin column
 - Collect DNA in 150µL of buffer



DFU Recovery

- The protocol includes 70 minutes of incubations not counting the germination plus the column purifications.
- Assuming 100 percent recovery, 10% (20) of the 200 CFU per filter make it to the extraction process. This number is increased by germination.
- A simpler method, shaking the filter in 4mL of buffer and doing a PCR directly on the supernatant gives a sensitivity of 3500 CFU/filter (our result). This protocol is used by a number of laboratories.
- Very few filters are inhibitory (and you can pretty accurately predict by appearance)
- Gain in the described protocol is around 20 fold over what we observe using a five minute protocol.



Qiagen: Purification of *B. anthracis* From Soil

- Qiagen recommends use of one of their kits for extracting soils
- Ten thousand *B. anthracis* detectable in 0.5 G sandy loam
- The 0.5 G is suspended in 900 μ L of liquid (~11 *B. anthracis*/ μ L)
- The liquid is processed using a robot and silica magnetic beads
- At the end of the process the specimen is eluted in 200 μ L, a 4.5 fold concentration assuming 100% recovery.
- The 2 μ L aliquots analyzed by PCR in the study would contain ~22 *B. anthracis* if the unprocessed sample were analyzed directly
- If diluted one to two or one to three to counteract inhibition we are still dealing with a detectable number



Summary

- The point of the preceding is not that the level of detection of a procedure is unimportant. A ten or twenty fold increase of sensitivity is crucial in many applications. Even more would be better.
- The problem is that this gain in sensitivity is hard won in time and expense.



Rapid Removal of Inhibitors From Forensic Samples

- An alternate method is not to purify the DNA but to remove the inhibitors
- A rapid method was incorporated into the Smith's Bioseq system used by first responders
- Sample collected onto surface of a filter and then forced through into a PCR tube containing dried reagents
- Convenient for setting up the reactions but not good at removing salts and material most commonly seen in the types of specimens normally mistaken for bioagents
- Much forensic material not particularly inhibitory and a properly formulated assay is quite robust



Inhibition of Swab Specimens Collected at Stepnogorsk

	Number	Percent
Number of specimens	1010	
Number inhibited	18	1.7
Number inhibited following dilution	5	0.5

Swab specimens added to buffer plus chelating agent and heated. Specimens added directly to PCR tubes and analyzed.



UNSCOM Sample Collection



B. anthracis in
Al Hussein Missiles



Detection of *Clostridium botulinum* in
R400 bombs required purification

- | | |
|---------------------|---------------------------|
| Transfer Vessel | <i>B. anthracis</i> |
| pH Probe Housing | <i>C. botulinum</i> A |
| | <i>C. botulinum</i> B |
| Centrifuge Rotor | <i>B. anthracis</i> |
| | <i>C. botulinum</i> A |
| Fermenter | <i>B. anthracis</i> (2) |
| Vials from Burn Pit | <i>B. anthracis</i> (2) |
| | <i>C. botulinum</i> A (3) |



Clinical and Veterinary

- Current procedures rely on immunodiagnostics and cultivation
- Molecular approaches have the potential to provide an accurate result more rapidly than culture
- The same basic protocols are used for extraction
- There is a range of specimen types of interest to the veterinary community



Clinical: Veterinary Pathogens

Virus	Family	Specimens of Choice (For USDA Approval)	Research Specimens of Interest
Foot-and-mouth-disease	<i>Picornaviridae</i>	Oral Swabs or Vesicular Lesions,	Probang, Cell Culture Supernatant
Classical Swine Fever	<i>Flaviviridae</i>	Nasal Swabs	Tonsil Scrapings, Cell Culture Supernatant
Porcine Reproductive & Respiratory Syndrome (PRRS)	<i>Arteriviridae</i>	Semen, Blood	Lymph Nodes, Other lymphoid tissue
West Nile	<i>Flaviviridae</i>	Blood, Neurologic Tissue, Bird Feces	Lungs, Heart
African Swine Fever	<i>Asfarviridae</i>	Will be validated on Nasal Swabs	Tonsil Scrapings
Rinderpest	<i>Paramyxoviridae</i>	pre-mortem: nasal & conjunctival swabs	Acute post mortem: spleen, lymph nodes, abomasum, tonsil and third eyelid
Bacterial	Family	Specimens (For USDA Approval)	Research Specimens
<i>Mycobacterium paratuberculosis</i>	<i>Mycobacteriaceae</i>	Culture confirmation, Feces	Milk
<i>Brucella</i>	<i>Brucellaceae</i>	Milk, Cheese, uterine discharges, aborted fetuses	Udder secretions, lymph nodes, testes or epididymes



Classical Swine Fever Virus (CSFV)

- RNA virus
- Highly contagious and often fatal disease of swine (Also known as hog cholera)
- Countries affected are banned from international trade of live animals and pork products

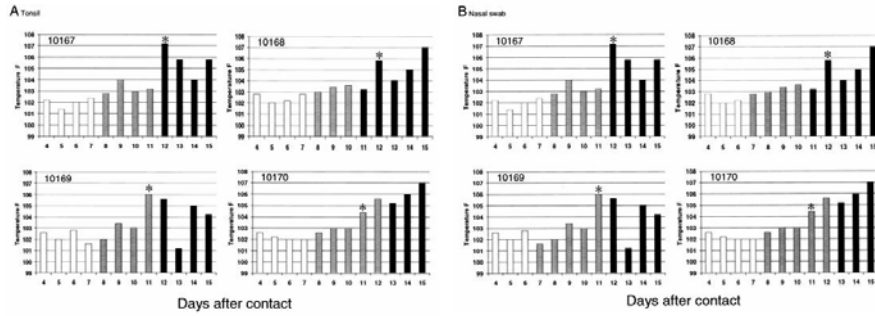


Diagnosis of CSFV

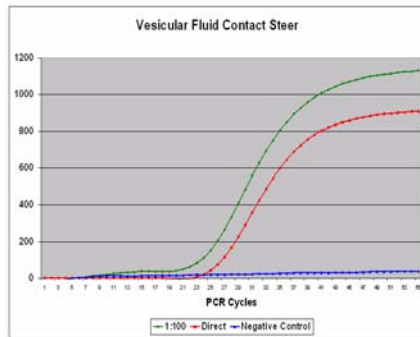
- Detection in tonsils by fluorescent antibody
- Antigen capture ELISA
- rtPCR
- Preferred specimen a nasal swab
- Our current test processes nasal swabs using standard column techniques



CSFV in Clinical Specimens Column Extraction



Detection of FMD Virus in Unprocessed Specimens



- Preferred specimens are oral swabs or vesicular lesions
- Processing might be required for one and not the other



Johne's Disease of Cattle

- Causative agent is *Mycobacterium paratuberculosis*
- Estimated that 7 to 8% of dairy herds and 22% of beef herds are infected
- Symptoms are diarrhea and rapid weight loss
- Might be linked to Chron's disease in humans



Difficulties

- Diagnosis by stool culture (8 to 16 weeks depending upon procedure)
- PCR from stool a much more rapid method
- Several grams of stool must be extracted to achieve desired sensitivity
- *Mycobacteria* extremely tough and must be physically disrupted to extract nucleic acids

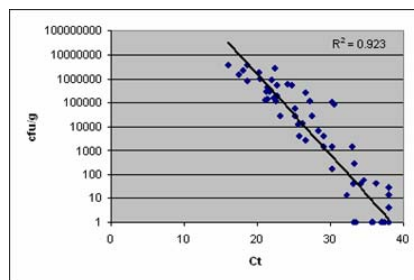


Mycobacterium paratuberculosis: Extraction Protocol

- Extraction protocol quite complex
 - Mix 2G fecal material with 35 mL buffer and allow to sediment
 - Remove supernatant
 - Beadbeat supernatant
 - Centrifuge
 - Purify DNA using spin columns
 - Because of large sample volume multiple applications to the columns are required



Johne's PCR Result



- The protocol is very effective Takes
- The graph shows a comparison of PCR result to colony count per gram of stool (frozen)
- USDA has granted a conditional license pending completion of results from nonfrozen manure
- The protocol would greatly benefit from a rapid, cost effective extraction method



Conclusions

- The goal is to find a few organisms in a large amount of material in a short amount of time at minimal expense
- One approach is to enrich for the organism, or at least to enrich for organisms of similar properties. A number of methods have been tried.
- Another is to increase the efficiency of nucleic acid extraction.



Purification Methods: Organisms

- Immunocapture
- Lectins
- Cationic and anionic exchange resins
- Nonspecific adsorption onto polymer beads
- Density gradient purification
- Electrophoresis/dielectrophoresis
- Culture
- Pelleting with metal hydroxides



Summary of Purification Methods: Nucleic Acids

- Phenol chloroform/ethanol precipitation
- Binding to silica
 - Spin columns
 - Beads
 - Microchips
 - Various fluidic chambers
- Binding to polymer beads
- Specific capture by probes



Conclusion

- The number of specimen types that must be analyzed is nearly equaled by the number of methodologies available to process them
- Very few of the systems are what we would consider high throughput and most are time and labor intensive
- A single technology that can handle all types with little “customization” would be extremely useful

