Developing a Sentinel-based Baja California Sur Rural Mexico Monitoring Program: Lessons learned from Alaska

Desarrollo de un programa de monitoreo basado en centinelas para el área rural de Baja California Sur: Lecciones aprendidas en Alaska

Abstract:

Alaska organizations [Alaska Native Tribal Health Consortium (ANTHC; main partner) and the University of Alaska Fairbanks (UAF)] participate in the Rural Alaska Monitoring Program (RAMP). The RAMP represents a major One Health project in the Department of Veterinary Medicine at the UAF and many units within ANTHC. The ANTHC has received most of the funding for RAMP from the US Department of Interior and the US Environmental Protection Agency. We utilize invertebrate and vertebrate sentinels to monitor environmental agents of diseases. Mosquitoes and whole blood soaked filter

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O'Hara, Todd*[1], Karsten Hueffer[1] Molly Murphy[1], J. Margaret Castellini[1], Yong Li[1], Lorrie Rea[2], James Berner[3]

[1] Department of Veterinary Medicine, University of Alaska Fairbanks, Fairbanks, Alaska (USA), tmohara@alaska.edu
[2] Institute of Northern Engineering, Water and Environmental Research Center, University of Alaska Fairbanks, Fairbanks (USA)
[3] Alaska Native Tribal Health Consortium, Division of Community Health, Department of Community Environment and Health, Anchorage, Alaska (USA)

papers (FP) from vertebrates are collected throghout Alaska, and elsewhere, by a network organized by our groups. Eluates (saline extracts) are prepared from dried FP collected by hunters and biologists for assessing infectious agent-specific antibody titers and other measures. We provide data on use for Toxoplasma gondii and Brucella spp. specific antibody titers via this standard technique and for a centrifugation method to concentrate eluate antibody for assay enhancement. Dried FP are also used for determining contaminants (mercury, polychlorinated biphenyls (PCBs)) and nutrients (selenium, Se) concentrations. Mosquitoes sent to UAF were tested by polymerase chain reaction (PCR) for presence of DNA from Francisella tularensis using three different genes. This technique can be easily adapted to other microbial organisms of interest. We report on these efforts to further develop our collaboration with colleagues in Baja California Sur (BCS) to implement similar procedures in remote areas with respect to agricultural and wildlife monitoring and research considering common concerns related to environmental change. The subsistence foods similarities and needs for environmental monitoring of agents important to Public Health fit well within this One Health paradigm whether one is addressing the subarctic or subtropic; thus enabling technology transfer and standardization as an overall trans-Americas One Health effort. The technology utilized in RAMP was presented in November of 2016 at the One Health of the Americas conference held in Todos Santos, BCS, Mexico so that we can develop a similar R(A)MMP (where one M represents Mexico and the A Alaska for our proposed collaboration).

Resumen:

Organizaciones de Alaska (Consorcio de Salud Tribal de Nativos de Alaska (Alaska Native Tribal Health Consortium, ANTHC), socio principal, y la Universidad de Alaska Fairbanks (UAF)) participan en el Programa de Monitoreo en el área Rural de Alaska (RAMP, por sus siglas en inglés). El RAMP representa uno de los proyectos principales abordando el tema de Una Salud en el Departamento de Medicina Veterinaria UAF y varias unidades dentro del ANTHC. El ANTHC ha recibido la mayor parte de los fondos para el RAMP del Departamento del Interior y la Agencia de Protección Ambiental de Estados Unidos. Utilizamos centinelas vertebrados e invertebrados para monitorear agentes ambientales de enfermedades. A lo largo de Alaska y otras zonas, se colectan mosquitos y filtros de papel (FP) empapados con sangre de vertebrados con apoyo de una red organizada por nuestros grupos. Se prepara un eluato (extracto salino) a partir del FP seco colectado por cazadores y biólogos para evaluar títulos de anticuerpos contra

agentes infecciosos específicos y otras variables. Proveemos datos del uso para títulos de anticuerpos específicos contra Toxoplasma gondii y Brucella spp a través de esta técnica estandarizada y para un método de centrifugación que concentra el eluato para mejorar el análisis de anticuerpos. El FP seco también se usa para determinar concentraciones de contaminantes (mercurio, PCBs) y nutrientes (selenio). Los mosquitos enviados a UAF se analizan por PCR para la presencia de DNA de Francisella tularensis usando tres genes diferentes. Esta técnica puede adaptarse fácilmente a otros microorganismos de interés. Reportamos sobre estos esfuerzos para desarrollar nuestra colaboración con colegas en Baja California Sur (BCS) para implementar procedimientos similares en áreas remotas con respecto al monitoreo e investigación en agricultura y vida silvestre considerando preocupaciones comunes asociadas al cambio climático. Las similaridades en alimentos de subsistencia y las necesidades de monitoreo ambiental de agentes importantes para la Salud Pública son acordes al paradigma de Una Salud (One Health) bien sea que nos enfoquemos en zonas subárticas o subtropicales; por lo tanto, permiten la transferencia y estandarización de la tecnología como un esfuerzo transversal en las Américas. La tecnología utilizada en RAMP se presentó en Noviembre de 2016 en el Congreso de Una Salud en América en Todos Santos, BCS, México para que podamos desarrollar un R(A)MMP (donde una M representa México y la A Alaska para nuestra propuesta de colaboración) similar.

Introduction

One Health

Most health practitioners are traditionally trained to work with a patient (the individual).

The individual is often the unit of concern and management. We do recognize there are those trained in family and population (herd) health that take a broader view and interventions. We are taking an even broader perspective when we approach health problems and solutions as "One Health" as practiced by the Rural Alaska Monitoring Program (RAMP) and proposed for this Baja California Sur (BCS) based Rural (Alaska) Mexico Monitoring Program (R(A)MMP), using invertebrates (mosquitoes) and dried vertebrate blood on filter paper (FP)[1] for monitoring environmental agents of disease. This approach very much fits within the Public Health infrastructure of BCS as there are many overlaps in mission and desired outcomes (pers. comm. Dr. R. Gaxiola-Robles). For this report "One Health" is recognized as:

"health for people, non-human animals, and environment is deeply connected and inseparable."

(http://source.colostate.edu/one-health-institute-opens-doors-colorado-state-university/)

Any person or group that is a steward of any of the components in the One Health paradigm is a potential "practitioner". One of our mutual concerns and collaborative research efforts between Alaska and BCS is the presence of mercury (Hg) in the fish-based food web (Gaxiola-Robles et al., 2014; Bentzen et al., 2014). We have va-

[1]Nobuto; http://www.advantecmfs.com/filtration/EnvironMonitor.shtml#nob

lidated the use of FP in the monitoring of Hg in blood of piscivores (Hansen et al., 2014) and look forward to using this technique in BCS. Alaskan and BCS colleagues would also exploit the collection of FP from BCS vertebrates for other agents of disease including some outlined in this paper.

Alaskan and BCS colleagues have been working together for many years on many One Health related research and monitoring efforts that include plants, invertebrates, and vertebrates; including human subjects. Many of these have focused on environmental contaminants and feeding ecology (Hernandez-Almaraz et al., 2016; Gaxiola-Robles et al., 2014; Bentzen et al., 2014; Barrera-García et al., 2013, 2012; Zenteno-Savin et al., 2013) but are easily amenable to monitoring other agents of disease, including infectious agents. We build on these efforts to propose a R(A)MMP for BCS by combining our experiences in Alaska with those in Mexico. We provide our method development and validation data here to kick start R(A)MMP; and propose foundation of Rural (Alaska) Mexico Monitoring Program (RAMMP).

The BCS-based entities for which we propose to build this relationship include Centro de Investigaciones Biológicas del Noroeste (CIBNOR), Hospital General de Zona No. 1, Instituto Mexicano del Seguro Social (IMSS), El Centro Interdisciplinario de Ciencias Marinas (CICIMAR), Universidad Autónoma de Baja California Sur (UABCS), and Colorado State University, Todos Santos Center (CSU TSC). The combination of these institutions and their associated expertise and resources makes R(A)MMP feasible. The likely inclusion of other groups would strengthen the proposed R(A)MMP. Those listed above are examples and we will encourage participation across many groups.

For this report "One Health" is recognized as: "health for people, non-human animals, and environment is deeply connected and inseparable."

Focus on zoonotic agents (vector and food) and chemical contaminants (food)

Within the R(A)MMP, the emphasis for assessing FP (blood) will be food-based pathways (organisms) of environmental agents to people; thus monitoring of species and matrices that are consumed. This includes wild caught and farm raised animals. For most of the chemicals of concern, we can utilize experts in chemistry and biomedical sciences at CIBNOR for measuring certain elements as conducted in Hansen et al. (2014) for total mercury (THg). In addition, we also propose to develop measures of essential nutrients so we can track the nutritional status of monitored animals (e.g., copper, zinc). Commercially available serologic assays (detection of disease agent specific antibodies) can easily be established at CIBNOR, UABCS, or CSU TSC for the specific agents of interest. We provide validation of FP collection and laboratory processing for use in traditional (standard) diagnostic assays, including a centrifugation approach to enhance sensitivity.

The emphasis on mosquito monitoring will be based on determining the best capture and

sampling methods for the region and the proximity to human and animal areas. This requires proof of principle testing for our colleagues in BCS. Once successful captures can be assured we will monitor these invertebrates for infectious agents of concern based on the availability of PCR-based methods for such agents as zika, chikungunya, dengue and yellow fever. Compared to the serologic tests mentioned above, establishing PCR-based methods may be more demanding on the facility resources in BCS (La Paz). Thus, we need to explore further where this can best be established for monitoring hundreds of invertebrate samples in an efficient manner with attention to high quality assurance (sequencing and informatics). One could consider use of marine invertebrates (e.g., shellfish) as well (Quakenbush et al., 2016).

As an example of the potential community-based scope of agents to consider, Sampasa-Kanyinga et al. (2012) documented exposure to zoonotic agents (Toxoplasma gondii, Toxocara canis, Echinococcus granulosus, Leptospira spp., Coxiella burnetii, and Francisella tularensis) in sera from 267 residents of Cree communities of James Bay (Canada). High seroprevalence rates were documented for Leptospira spp. (23%), Francisella tularensis (18%), and Toxoplasma gondii (9%). Seroprevalence rates of less than 5% were observed for Coxiella burnetii, Echinococcus granulosus, and Toxocara canis. It should be emphasized this relates to exposure only, and no obvious adverse outcomes were associated with these findings. However, Public Health officials should recognize exposure potential and that continued hygiene and food safety awareness are critical to prevention of disease. This effort has enhanced value in the face of climate change and the enhanced movement of agents among the Americas (e.g., trade, biota, military operations, resource extraction industries, tourism).

Shared pathogens of concern

There are numerous agents and organisms of mutual concern for our subarctic and subtropical environments. For example, Brucella spp. is a zoonotic agent present in wildlife and agricultural species for which the FP technique has been validated and used (Curry et al., 2011A, B) and is a pathogen of interest in BCS. This organism represents a significant wildlife and livestock agent of disease that can impact health and reproductive outcomes and the management of wildlife and agricultural resources between states and countries. We plan to develop this Brucella spp. antibody monitoring capability, including reconstituting the eluate to a protein concentration similar to plasma or serum, as an initial effort in BCS.

We present data on Toxoplasma gondii as well. As viruses are a global concern, the FP technique has been used to detect antibodies to agents including distemper and parvovirus (Kamps et al., 2015), West Nile Virus and five bovine viruses (Curry et al., 2014), and Avian Influenza Virus (Dusek et al., 2011) and thus is proven in this regard and can be applied to specific viral agents of concern.

We present basic methods, preliminary data, and some discussion of the approaches planned for establishing a disease agent monitoring program for BCS that

utilizes invertebrates and blood soaked Nobuto FP based on our success in our Alaska-based RAMP.

This approach will include a One Health perspective that must involve graduate and veterinary medical students from the "Americas" so we can advance our understanding of disease issues in a rapidly changing world (e.g. climate, technologically, culturally, politically, economically, etc.).

Methods

Basic methods of capture and collection (vertebrates)

Samples are easily collected once blood is recovered from the animal by simply using a syringe (apply blood to the Nobuto filter paper strip so as to absorb the estimated 100 µl). One can also soak the FP in the tube of blood. It is important to assure the blood is homogenous and that the FP is properly soaked and air dried. Some pinniped samples were collected for diagnostic purposes for animals admitted to The Marine Mammal Center (TMMC) for rehabilitation. Sample collection methods and handling are reviewed in Hansen et al. (2014) and Van Hoomissen et al. (2015).

Samples from wild pinnipeds brought to TMMC (Sausalito, California, USA) were sampled under authority of Marine Mammal Protection Act permit 932-1905/MA-009526. Harbor seals (Phoca vitulina) from Alaska were sampled in accordance with approval of Institutional Animal Care and Use Committees at the University of Alaska Fairbanks (protocol 07-37) and the State of Alaska Department of Fish and Game (Gail Blundell, protocol 07-16), as well as a permit from the National Oceanographic and Atmospheric Administration under the Marine Mammal Protection Act (Permit 358-1787-00 to Gail Blundell) as reported in Hueffer et al. (2011 and 2013). For Steller sea lions (Eumetopias jubatus) samples were collected in a manner similar to that reported in Peterson et al. (2016) as part of research efforts to assess range wide concentrations of mercury and selenium in sea lion pups permitted under MMPA permit 14326-02 issued by NOAA, and IACUC protocols at UAF (protocol 594759-2) and NOAA (protocol NW2103-2). Bottlenose dolphins (Tursiops truncatus) were live-captured, sampled, and released after health assessments in Sarasota Bay, Florida during May and July 2012 by staff from the Chicago Zoological Society (National Marine Fisheries Service Scientific Research Permit 15543, Institutional Animal Care and Use Committee 11-09-RW1) under supervision of principal investigator Dr. Randall Wells.

For recently dead animals, collection of free flowing, or other forms of uncoagulated blood, is feasible. Simply soaking the FP should accomplish proper sampling if one avoids clotted blood or blood mixed with other fluids or sources of environmental contamination. The FP samples are then air dried and placed in paper envelopes.

Analytical Chemistry (Hg and Se; PCBs)

The methods for measuring concentrations of total mercury ([THg]) and total selenium ([TSe]) are well described in other publications, thus for the sake of space we refer readers to those more detailed documents: Hansen et al. (2014), McGrew et al. (2015), Co-

rrea et al. (2015), and Van Hoomissen et al. (2015). The blank FP samples (Nobuto strips, not blood soaked) were provided to the Applied Science, Engineering, and Technology (ASET) Laboratory at the University of Alaska for polychlorinated biphenyls (PCBs) analysis by gas chromatography (GC; Agilent 7890 GC) utilizing Electron Capture Detection (ECD). This method provides determination of 18 PCB congeners.

Mosquito Sampling

Mosquitos were obtained using battery operated CO_2 generating traps (Mosquito Magnet), placed in proximity to freshwater bodies, within 0.25 km of rural Alaska Native villages on the Bering Sea coast, during the months of May-September. The device desiccates the trapped mosquitos. Samples were stored in Whirlpaks at ambient temperature until being shipped for PCR analysis.

Screening for Francisella DNA in mosquitoes

We screened for Francisella in mosquitoes collected in rural western Alaska using Polymerase Chain Reaction (PCR) based methods. We established three real time quantitative PCR methods at UAF designed to detect DNA of three Francisella genes: lpnA2, fopA, and iQFt1. These three assays have average detection limits of 36, 825 and 2680 genome copies per reaction for lpnA2, iQFt1, and fopA, respectively. Based on these findings we pool 5 mosquitoes into one DNA extraction and screen the obtained DNA with the most sensitive assay (lpnA2) and confirm positive samples with the other assays. Based on results, mosquitoes remaining from the original sample can be screened and positive samples can be identified to species using DNA barcoding technique as described previously (Triebenbach et al., 2010).

Serology

Toxoplasma

The Georgia Veterinary Diagnostic Laboratory tested serum using their standard latex agglutination test. This test detects IgG and IgM antibody to Toxoplasma gondii. The positive threshold value used is a titer of 32 or greater. However, we do not attempt any diagnostic interpretation as we are simply using this test (measured titers) to determine the efficacy of our centrifugal eluate reconstitution technique that is described elsewhere.

Brucella (Card and Plate tests)

For the card test we used 3 categories of responders: 1) Strong Positive (++) - large, numerous clumps. Nearly identical to positive control, 2) Moderate Positive (+) - clumping, but not as numerous or as large as full positive results, and 3) Minor agglutination/Negative (-) – either no agglutination present or fine, slight agglutination present but not enough for a moderate positive designation.

This allowed for some assessment of "strength" of response but is not as discrete or useful as determination of titers as conducted in the plate test. For further details, see Hueffer et al. (2013). Positives on the plate test were determined by the presence of any agglutination at each titer level tested (25, 50, 100, 200, 400). For further details, see Hueffer et al. (2013).

Eluate and centrifugal reconstitution (protein concentrating)

Elution Methodology

The serum or whole blood soaked FP were eluted similar to Hansen et al. (2014). Each FP was cut into five to seven pieces and placed in a 2ml cryogenic tube then 400µl of PBS with 1% penicillin–streptomycin was added. The tubes were then agitated using a vortex mixer to ensure that each FP segment was soaked in the solution then eluted overnight (~16 hours) at 4°C.

Each tube, containing one FP, yielded approximately 200 µl of eluate. According to the FP manufacturer's specifications elution of whole blood by this method has been estimated to be a 1:10 dilution from serum. When we used FP soaked with serum instead of whole blood, the dilution of our eluate was estimated to be about 1:5 from serum. Two FP eluates from each animal were combined, to be used in a non-concentrated form in the subsequent testing.

Reconstitution Methodology (centrifugal)

The goal is to concentrate the eluate to match original serum composition as closely as possible. We are mostly interested in reconstituting the activity (titer level) of antibody reactivity in commonly used immunoassays. For example, by concentrating about 1000µl of eluate (eluates of 5 FP strips combined) to about 250µl, we would aim to approximate the original serum. Prior to beginning the concentration protocol, 300µl of MES (2-(N-morpholino) ethanesulfonic acid)) was added to each Amicon centrifugal filter (Amicon® Ultra Filter) and left to incubate overnight at 4°C. Further processing followed manufacturer instructions.

Results and Discussion

Mosquito based monitoring

Mosquitoes were tested for Francisella tularensis (Ft) for two reasons: First the bacterial pathogen, suspected to be present in rodent species which have extended their range north in Alaska, as the tree line has moved north, can be transmitted by mosquitoes; and secondly as blood feeding arthropods mosquitoes contain host blood that may contain pathogens regardless of the competency as a vector for the pathogen in question. Human infection (tularemia, mild to life-threatening based on route of exposure and expression in host) with Francisella tularensis has been occasionally documented in the north; transmission by mosquitos has never been investigated in North America but has been documented in Europe (Hansen et al., 2011). Especially with environmental change and expansion of beavers and other possible reservoirs of Ft, this pathogen is of special concern in the subarctic. However, our methods can be easily adapted to subtropical climates by adjusting the pathogen assayed for including zika, dengue, chikungunya, malaria, and many others that are DNA-based and found in the circulation.

Blood sampling benefit

Assessment of whole blood in itself can provide a great deal of information about the health of an animal and when used collectively for a group (e.g., herd, flock, stock,

haul-out) can be used to assess population health. Whole blood can provide information outside of the traditional clinical uses. Here we consider whole blood as a matrix to assess "exposure". We are referring to "exposure" to certain chemicals such as contaminants or essential nutrients.

In addition, we assess historic, and current, exposure to certain infectious agents of disease as well as specific antibodies generated to these agents. The detection of agent specific RNA and DNA indicates current presence whereas agent specific antibodies can relate to past exposure, possibly ongoing exposure as well.

Components of blood are often used to provide more specific information. In some cases, we only need the fluid compartments. Serum (clotting factors removed) and plasma (anticoagulant treated, clotting factors present) are common, but different representatives of the circulating fluid (acellular) fraction. The cellular fraction of blood can be quite useful as well and can be assessed as whole blood but in some circumstances the cells, including specific cell types, are removed for assessment. We do not go into detail here but want the reader to appreciate the complex nature of blood (e.g., various cell types and multiple fluid components). This is important when considering the use of whole blood soaked FP (cells present), or using serum soaked FP (cells removed), as presented in this report.

For whole blood soaked FP we can no longer separate the compartments identified above. Cells have lysed and their contents are now a part of any extraction from the dried FP; except for those bound to the paper. Thus, when we prepare the whole blood eluate in PBS we must consider it is liquid but does NOT represent plasma or serum. Thus, the utility of the eluate is not directly comparable to the commonly used fluid compartments of blood (serum, plasma) for many analytes and assays. Depending on the analyte or endpoint the use of the eluate or the blood soaked FP directly can be considered. For some measures, the presence of lysed cells contents is not conducive to this sampling technique whereas for others, such as antibodies, we can effectively use the eluate to measure antibody titers. Thus, some of our efforts have focused on validation of these procedures for this complex eluate.

Non-blood FP sampling benefit

Above we describe the use of Nobuto FP for use with whole blood or serum. We will provide examples from our work in Alaska below. One of our key missions in BCS is to validate the use of this technology on non-blood matrices for use in agricultural, and possibly wildlife, scenarios. There are key diagnostic tests that use non-blood fluids such as the chemistry or microbiology of urine or milk.

We collected intact fluids and fluid soaked FP as matched samples from individual animals for parallel testing using the intact fluid as the gold standard to determine if the FP sample provides similar information. For milk, we can assess nutrients and contaminants using standard chemical tests, and for zoonotic agents of interest we can explore antibody based tests and direct measure of microorganism constituents (e.g., DNA or RNA). This is proposed as student-based research projects that would be based out of La Paz (BCS).

PCR-based methods for FP

We used three different genes (lpnA2, fopA, and iQFt1) to determine the presence of Francisella DNA in these insect pools. In a survey of pooled mosquitoes from the Fairbanks area, 30% of the pools were PCR positive for Ft. For rural Alaska, 9 pools out of 56 total pools (5 mosquitoes/pool) consistently tested positive for lpnA2 (best detection level at 36 genome copies/reaction of the three genes). We conducted validation studies and we are analyzing samples from field efforts as part of further establishing this monitoring effort. This technique is ready for transfer to BCS scenarios.

Even though it is not a part of RAMP, we point out that host DNA can be characterized from these samples as well. This might be important in some situations. However, using these specific FP types likely has limited use in gene expression studies as RNA is not easily stabilized and preserved as compared to DNA. With respect to future efforts and validation we are investigating the use of blood soaked materials for assessing genomic and transcriptomic assessments.

Serological-based methods for FP

We are not compelled to do an expansive discussion on the serologic use of the FP as that is what they are designed for and many researchers have shown their value in this regard (Curry et al., 2011A, B, 2014; Kamps et al., 2015; Dusek et al., 2011). However, we do emphasize that we have introduced a technique that can reconstitute the eluate to approximate antibody titer levels of serum/plasma (Amicon® Ultra Filter).

Using assays of pathogen specific antibodies, we have shown increased titers in concentrated eluates that are close, or identical to, matched serum samples (the saline extracted eluate is dilute relative to blood, serum or plasma).

This post eluate processing allows for more relevant testing for assays that determine positivity at relatively low titers; such 1:2, 1:4, or 1:8, or where one wants to optimize detection. We use two agents of concern (Toxoplasma gondii and Brucella spp.) and standard diagnostics tests (see below) to compare a gold standard (serum or plasma) to the eluate from the FP and eluate that is centrifugally treated to concentrate protein to approximately what would be expected in serum or plasma. We were graciously provided samples from populations known to be exposed to these microorganisms so that we could investigate this approach with a high expectation to find pathogen specific reactive antibody in the samples.

Toxoplasma

Titers for antibodies to Toxoplasma gondii from bottlenose dolphins (Tursiops truncatus) from Sarasota, Florida (USA), a population known to be exposed to this organism, are listed in Table 1 for each individual dolphin tested (n=16 individuals, with duplicate plasma and WB FP eluates prepared for 9 of the 16; i.e., 25 total matched pairs of plasma and WB FP eluates). These are presented with respect to our gold standard matrix, the WB FP eluate, and the WB FP eluate post centrifugation (reconstituted). We highlight that 13 were negative as plasma sam-

ples (10 individuals with at least one negative sample) with 12 WB FP matched eluates as negative (9 individuals with at least one negative sample; one positive at 1:16, FP 057 and dolphin ID FB# 252). Within Table 1 we highlight as bold (FP0XX) animals that indicated reconstitution of the gold standard titer to within one dilution post centrifugation (e.g., successful reconstitution).

Table 1. Titers for antibodies to Toxoplasma gondii from bottlenose dolphins (Tursiops truncatus) for gold standard (Plasma Titer), the whole blood (WB) FP eluate (WB Eluate titer), and the WB FP eluate post centrifugation (Concentrated WB Eluate Titer). Highlighted as bold (FP0XX) animals that indicated reconstitution of the titer to within one dilution post centrifugation (e.g., successful reconstitution). Two values for a matrix indicate duplicate samples from the same animal.

Sample	Dolphin ID FB#[a]	Plasma Titer	Concentrated WB Eluate Titer	WB Eluate titer
FP 054, 078[b]	142	Neg, Neg	Neg, Neg	Neg, Neg
FP 051	164	Neg	Neg	Neg
FP 059	173	Neg	Neg	Neg
FP 060	232	Neg	Neg	Neg
FP 052, 064	233	Neg, Neg	Neg, Neg	Neg, Neg
FP 046, 070	274	Neg, Neg	Neg, Neg	Neg, Neg
FP 061	278	Neg	Neg	Neg
FP 049, 075	20	Neg, 16	Neg, 16	Neg, Neg
FP 057, 073	252	Neg, 16	16, 16	Neg, Neg
FP 050, 076	159	Neg, 64	Neg, 16	Neg, Neg
FP 047, 079	113	16, 16	Neg, Neg	Neg, Neg
FP 048, 074	258	16, 64	16, 32	Neg, Neg
FP 058	7	32	32	Neg
FP 056	221	64	64	Neg
FP 053	242	2048	4096	512
FP 055, 077	276	4096, 8192	4096, 4096	512, 512

[a] ID number assigned to each individual dolphin

[b] For some individuals (e.g. FB# 142) there were duplicate pairs of plasma and WB FPs prepared (e.g. FP 054, FP 078). Titers from matched plasma and FP eluates are listed in the order of the corresponding FP number for that individual.

This represents 9 samples out of the 12 total number of samples with a measured titer in plasma (7 out of 9 individuals), or 75% of samples were returned to a titer close to, or the same as, the gold standard. Dolphin FB# 159 (FP 076) did not regain the reactivity based on plasma results; plasma samples from dolphin FB# 113 (FP079 and FP047) were at the lowest recorded titer for plasma thus results are inconclusive for the concentrated eluates as negatives. We conclude that the centrifugation technique enhances the use of blood soaked FP with respect to measuring T. gondii specific antibodies and that this would likely apply to other agent specific antibodies.

Brucella

Prevalence of Brucella reactive antibody is approximately 13/80 (16.3%) and 14/79 (17.7%) for the card and plate test, respectively, for this group of harbor seals from Alaska (Table 2A). Brucella card and plate agglutination tests are not amenable to using WB FP eluate so these comparisons were conducted using eluates of serum soaked FPs. However, other assays can likely be used that do not rely on agglutination as a measure.

Table 2A: Brucella card and plate test results for harbor seal serum.

	Positive	Negative
Card	13	80
Plate	14	79

For the card test (Table 2B), the 7 serum negative samples were also negative for the FP Eluate-concentrated and FP Eluate samples (control). For the 5 samples

classified as Serum ++ [serum samples showing strong reactivity]: 2 of 5 were reactive (1 at ++ and 1 at +) for FP Eluate and 4 of 5 were reactive (1 at ++ and 3 at +) FP Eluate-concentrated. Thus, reconstitution (centrifugation) resulted in 4 of the 5 being (remaining) positive. For the 8 weaker serum responders (+) only one sample remained reactive as FP Eluate whereas 5 samples remained + for FP Eluate-concentrated. This indicates concentrating these samples helps to retain antibody reactivity as compared to eluate alone for the card test. Although the FP method would still provide a lower prevalence estimate as compared to the serum samples, it would still show an exposure to a zoonotic agent is very likely for this group of harbor seals at the population level. In summary for the card test, serum showed 13 positives of which eluate only detected 3 of 13 while the centrifugally concentrated eluate detected 9 of 13 (7 negative controls remained so). Thus, we tripled the number of detectable samples with centrifugation and we conclude this technique enhances Brucella-specific antibody detection for the card test when using blood soaked or serum soaked FP.

Tables 2B and 2C: Comparison of serum with serum soaked FP eluate and concentrated eluate for detection of Brucella reactive protein using Card Test (Serum 13++ or +, 7-) and Plate Test (Serum 11++ or +, 9-). Positives on the Card Test were determined by degree of agglutination, while positives on the Plate Test were determined by the presence of any agglutination at specific titer levels (25, 50, 100, 200, 400+).

Table 2B Card Test	# Occurrences		
[a]Serum ++	Serum	FP Eluate$_{concentrated}$	FP Eluate
	5 ++	1 ++	1 ++
		3 +	1+
		1 -	3-
	Total Positive	4/5	2/5
[b]Serum +	8 +	1 ++	0 ++
		4 +	1 +
		3 -	7 -
	Total Positive	5/8	1/8
[c]Serum -	7 -	0 ++	0 ++
		0 +	0 +
		7 -	7 -
	Total Positive	0/7	0/7

For the Brucella plate test (Table 2C), the 9 negative samples used remained negative for both the FP Eluate-concentrated and FP Eluate. For the 5 ++ samples 5 of 5 and 2 of 5 samples remained positive for the FP Eluate-concentrated and FP Eluate, respectively. This indicates that reactivity was maintained post FP processing for both approaches. However, for the 6 serum + samples reactivity was lost in that only 1 and 0 retained reactivity for the FP Eluate-concentrated and FP Eluate, respectively. This indicates a post FP processing loss of reactors (Table 2C). In summary, serum showed 11 positives of which eluate only detected 2 of 11 while the concentrated eluate detected 6 of 11 (9 negative controls remained so) for the plate test. We conclude this concentration technique enhances Brucella-specific antibody detection for the plate test as we tripled the number of detectable samples with centrifugation while no decrease in specificity was detected as all negative samples remained negative after concentrating.

Table 2C Plate Test	# Occurrences		
[d]Serum ++ (≥400)	Serum	FP Eluate$_{concentrated}$	FP Eluate
	5 ++	2 ++	1 ++
		3 +	1 +
		0 -	3 -
	Total Positive	5/5	2/5
[e]Serum + (25≤x<400)	6 +	0 ++	0 ++
		1 +	0 +
		5 -	6 -
	Total Positive	1/6	0/6
[f]Serum -	9 -	0 ++	0 ++
		0 +	0 +
		9 -	9 -
	Total Positive	0/9	0/9

[a] Card test - Strong Positive (++) - large, numerous clumps. Nearly identical to control.
[b] Card test - Moderate Positive (+) - clumping, but not as numerous or as large as full positive.
[c] Card test - Minor agglutination/Negative (-) – Either no agglutination present or fine, slight agglutination present but not enough for a moderate positive designation (i.e. +).
[d] Plate test - Strong Positive (++) – agglutination at titers ⩾ 400
[e] Plate test - Moderate Positive (+) - agglutination at titers between 25 and 400
[f] Plate test - Negative (-) - no agglutination present at titer = 25

Chemical-based methods

Our group, and many others, have established the value of using blood soaked filter paper (Nobuto and other) for determining presence and concentrations of some chemicals of interest (Hansen et al., 2014; Jantos et al., 2011; Li et al., 2010; Alfazil and Anderson et al., 2008; Stanton et al., 1999; Spliethoff et al., 2012; Stove et al., 2008; Burse et al., 1997). In our laboratory at the UAF, we successfully showed that we could reliably measure total Hg concentrations ([THg]) in 3 FP products. Considering we were able to directly analyze the blood soaked FP (directly burn the FP and measure the Hg content with no processing), that matrix was deemed the most efficient and accurate measure that matched very well with corresponding whole blood samples (Hansen et al., 2014). We continue to validate this sampling technique for other chemicals of interest (selenium, stable isotopes of C and N) that we have not yet published. We provide additional data here on continued work with THg, preliminary data on PCBs and total Se concentrations ([TSe]). For our R(A)MMP efforts we plan to include essential elements in the scheme so that we can also address nutritional adequacy or deficiency. Based on our success in measuring elements (e.g., Hg) on FP (Hansen et al., 2014) we anticipate success with other elements. Regardless, we will conduct the needed validation studies for this application in BCS, Mexico.

Preliminary PCB findings FP alone

We hypothesized that Advantec Nobuto filter paper can also be used to quantify PCB concentrations in whole blood. We selected this as an easily measured organohalogen for this initial assessment. Our goal was to determine whether the FP had low enough blank background [PCB] for this use, indicating promise for this approach when soaked with blood and then extracted. If the strips provided inherently high background levels, then there is no reason to pursue this method further for the relatively low concentrations of PCBs

one could anticipate in blood. The majority of the PCB recovered from the blank strips (data not presented) detected only PCB 31 across the samples analyzed. In general, the higher the numbers of blank strips in the sample, the higher PCB 31 concentration detected. For example, the 20 strips sample recovered the highest amount of PCB31 levels out of all of the other samples. This general trend occurs with PCB 52 yield as well; whereas only 15 FP blanks and 20 FP blanks measured PCB 52 above the detection limit (using fewer strips were below detection but we can assume PCB52 is still present). Based on the results of these extractions, we can conclude that FPs do have a low enough background (exceptions PCB 31 and PCB 52) and might recover detectable concentrations of PCBs. However, we caution that a single FP holds approximately 100µl of whole blood and this low volume is likely inadequate for reliable concentration measurements that are derived from whole blood. Thus, we are presented with a dilemma of using multiple strips to achieve a reasonable volume (e.g., 200 to 500 µl) to detect what is in whole blood while simultaneously increasing the background levels by using more strips as shown for PCBs 31 and 52 (data not presented). We continue to explore how we can determine organohalogen contaminants in whole blood soaked FP without introducing a high background level or requiring an unreasonable number of FPs for each analysis.

Total selenium concentrations ([TSe]) for FP and Whole Blood

Preliminary data using matched whole blood and WB soaked FP from Steller sea lions (SSL) suggest that WB FP may be useful for assessing whole blood Se status ([TSe], Figure 1. The linear relationship is strong (r2 of 0.8039) and very much parallels unity (slope of 1.1903). As expected, there is more innate variability in the analysis of [TSe] than [THg], with recoveries from certified reference material typically from 85-115% (compared to 95-105% for [THg]). This analytical variability will result in more variability when comparing [TSe] in samples from the same individual as compared to [THg]. Nevertheless, these preliminary data show a strong relationship between [TSe] in whole blood and WB FP. We will follow up using whole blood and WB FP samples from harbor seals, northern fur seals (Callorhinus ursinus) and other pinnipeds to determine if this strong relationship persists. We plan to directly apply this method to measure of [TSe] in blood of animals sampled in BCS as well as attempt to measure [TSe] in other matrices.

Comparison of whole blood, blood soaked filter paper (FP), and hair [THg]

We have validated use of FP based assessments of [THg] by comparing it to matched whole blood samples (Hansen et al., 2014) so one can estimate circulating levels of Hg. In our next phase of validation, we are assessing Se (results presented above) as it is well known to be associated with [THg] and is a key antioxidant. Also, we are determining how whole blood and FP-based [THg] compares to hair [THg] as both matrices are important for assessing Hg exposure but integrate Hg in different ways that affect interpretation. We are promoting the sampling of full length hair and FP as a part of RAMP and R(A)MMP. Some of our

previous work related to hair and blood [THg] comparisons are presented in Peterson et al. (2016). Thus, combining these matrices will allow for a more complete temporal assessment where hair integrates Hg over time (about 1cm of growth every month for humans) and whole blood Hg assessments represent current status and likely recent dietary exposure.

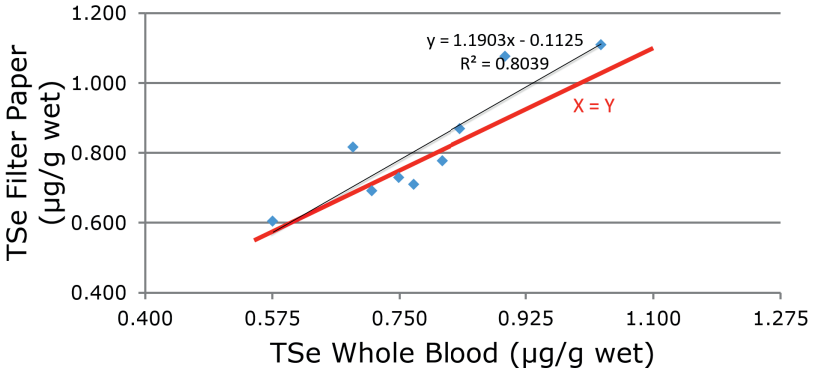

Figure 1: Comparison of total selenium (TSe) concentration between whole blood (TSe Whole Blood (µg/g wet)) and blood soaked filter papers (TSe Filter Paper (µg/g wet)) in Steller sea lions.

Each has value and when combined gives a much more complete picture of the Hg exposure status. Collection of full length hair and blood soaked FP is likely to be an important One Health monitoring approach that is inexpensive and logistically feasible under most field conditions.

By validating THg and TSe measures in blood soaked FP we can compare the determination of the TSe:THg molar ratio in blood (not hair) that we often use to relate Se status to Hg concentrations for assessing potential "imbalances" for these interacting elements (Correa et al., 2015; Quakenbush et al., 2016). These approaches will be implemented in BCS (Mexico) and have particular value to fish eating organisms including humans.

We have extended validation studies of [THg] in blood soaked filter papers in additional pinniped species using matched blood soaked FP and whole blood from California sea lions (Zalophus californianus), elephant seals (Mirounga angustirostris) and additional harbor seal samples from The Marine Mammal Center (TMMC) in Sausalito, CA (Figure 2).

TMMC colleagues have strict clinical protocols for sampling that allow us to do high quality diagnostics and validation assessments that we can then combine with more remote sampling by biologists and hunters in Alaska (e.g. data presented above for Brucella serology). These data confirm the conclusions of Hansen et al. (2014) and validate the use of blood soaked filter papers for [THg] assessment across a range of species that we plan to integrate with use of hair samples (e.g., short and long term assessments of Hg exposure) in a future publication. The [THg] linear relationships (whole blood compared to FP) were quite impressive for harbor seals and California sea lions ($r^2 = 0.9655$ and $r^2 = 0.9826$; respectively) while elephant seals show a promising relationship based on r^2 ($r^2 = 0.8038$, $p<0.05$) that may require more investigation with all 3 species showing significant linear relationships ($p<0.05$).

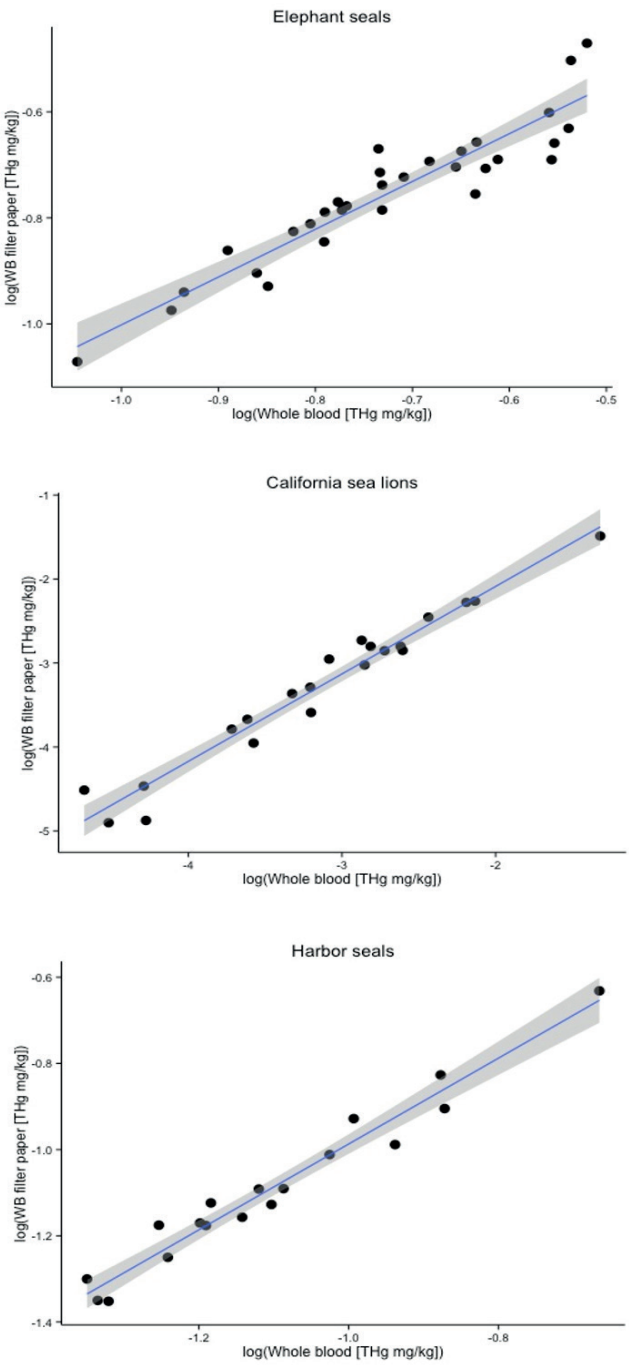

Figure 2: Linear regression comparing total mercury concentrations ([THg]), converted to wet weight between whole blood and blood soaked filter papers (FP) from three pinniped species.

Conclusion

The PCR-based methods have shown utility in our vector sampling scheme to detect bacteria that we will expand in scope in Alaska and initiate in BCS. We have validated the use of these blood soaked strips to detect agent specific antibodies that was enhanced with a centrifugation technique to reconstitute the samples back to concentrations found in the gold standard matrix (serum).

This indicates we can achieve a reasonable level of sensitivity to determine the presence of exposure to certain agents of disease. We will implement this in BCS in the very near future. Chemical analyses of blood soaked FP for elements in blood shows great promise if we use Hg and Se as our prototypical elements. We will explore use in other fluids such as milk and urine in BCS for some of the measures validated with use of blood and serum. The Alaska experience (RAMP) should allow for establishment of R(A)MMP in BCS based on common interests in addressing climate driven changes in the environment in a One Health context.

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