THESIS

QUANTIFYING BIOMARKERS IN WILDLIFE EXPOSED TO LOW DOSES OF ENVIRONMENTAL RADIATION PILOT STUDY

Submitted by

Nadia Halim

Department of Environmental and Radiological Health Sciences

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Master's Committee:

Advisor: Thomas Johnson

Alexander Brandl
James Lindsay

ABSTRACT

QUANTIFYING BIOMARKERS IN WILDLIFE EXPOSED TO LOW DOSES OF ENVIRONMENTAL RADIATION PILOT STUDY

Exposure of free-ranging wildlife to environmental radiation is of concern following the nuclear accident at the Fukushima-Daiichi facilities in 2011. The uncertainty associated with exposure to chronic ionizing radiation in the vicinity of the accident continues to concern the general population, as well as produce seemingly conflicting scientific results. The risk from prolonged, low dose/low dose rate radiation exposures, specifically to wildlife, remains relatively uncertain. The quantification of chromosomal aberrations such as dicentrics and micronuclei was evaluated as a method of estimating radiation dose to wild boar. Dicentrics and micronuclei found in blood samples of humans are known as a biomarkers of radiation exposure. Blood samples were collected from wild boar in two towns in Fukushima prefecture in Japan and from Kentucky in the USA. External dose was also estimated using soil sample analysis. As a pilot study, only the feasibility of using dicentrics and micronuclei to estimate radiation dose in wild boar was investigated. Additional studies will be required to ascertain the suitability of measuring other chromosomal aberrations and/or decreased telomere length as a method of ascertaining wild boar radiation dose. The hypothesis of the pilot study was that it is possible to estimate chronic radiation dose to wild boar exposed to low levels of lingering environmental ionizing radiation in Fukushima prefecture as well as in irradiated blood from wild boar residing in areas

experiencing only natural background radiation with biodosimetry techniques. The data obtained from this investigation do not prove the feasibility of using dicentrics and micronuclei formation to estimate wild boar radiation dose. While the technique for processing wild boar blood in order to observe chromosomal aberrations was successful, the levels of radiation exposure to the wild boar were too low and did not produce biomarkers for use as an indicator of internal radiation dose indicating the hypothesis to be incorrect. Other methods of estimating low radiation dose to wild boar will need to be investigated in future studies.

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INTRODUCTION

Long-Term, Low Level Radiation Exposure

Due to the interest in and lack of data regarding the effect of long-term, low-level radiation dose, a considerable amount of public and governmental concern revolves around any and all human exposure to radiation. Beginning with the research supported by the Manhattan District to researchers at Argonne and Oak Ridge, who began studies before 1943, there has been interest in the effects of low-level, long-continued irradiation from external sources and the tolerance of man to the irradiation (Brues et al.1959). There are available data on the human biological effects of short-term, high-dose radiation exposure from multiple radiation incidents, such as the atomic bomb survivors, and accidents in the twentieth century. While radiation worker studies where workers have been exposed to low levels of radiation are very informative due to the objective measurement of a large range of cumulative doses through personal dosimeters, the studies are subject to important limitations such as statistical uncertainty and potential confounding (Gilbert, 2001). The limitations make the worker studies unadvisable for replacing atomic bomb survivors as the primary source of data for cancer risk estimation from radiation exposure (Gilbert, 2001). Most of the current data analyses involving human exposure to low levels of radiation (both acute exposure and long-term exposure) have been from the atomic bomb survivors in Japan in 1945 and the Chernobyl nuclear accident in 1986.

The atomic bomb survivors' radiation dose estimates have been carefully evaluated and all the health effects in comparison to the radiation doses received have been analyzed and included in the BEIR VII report (BEIR VII). Careful analysis of the atomic bomb survivor dose versus cancer incidence data has indicated that the overall occurrence of solid cancers increases in proportion to radiation dose (BEIR VII). However, the specific amounts of radiation dose for which any type of cancer can or will occur in the individuals exposed to radiation are unknown due to lack of data. The rate of incidence of specific cancers in the atomic bomb survivors has been analyzed and deviances from the rate of incidence for cancers within the general population have been noted. The incidence of cancer has been higher in the atomic bomb survivors than in the general population (BEIR VII). Also, in the BEIR VII report, Preston and colleagues (2003) presented lifetime cancer risk estimates for atomic bomb survivors exposed to 1 Sievert (Sv) of radiation. For a person exposed at age 10, the lifetime cancer risk estimate was 18–22%, 9% for a person exposed at age 30, and 3% for a person exposed at age 50 (BEIR VII). The BEIR VII report attempts to develop the best possible risk estimate for human exposure to low-dose, low-LET radiation from the collection and analysis of data of individuals exposed to low levels of radiation. The BEIR VII report also develops risk estimates from exposure to higher levels of radiation. The three different models that have been argued to be used to depict low-levels of radiation exposure are the threshold model, the linear-quadratic model, and the linear no threshold model (LNT).

While there is discussion in the BEIR VII report regarding how the LNT model best represents the low-dose human exposure to radiation by depicting how the risk of cancer proceeds in a linear fashion at lower doses without a threshold and how the smallest dose has the potential to cause a small increase in risk to humans (BEIR VII, 2006), there is not enough scientific evidence proving the validity of the LNT model as the atomic bomb survivors are too small of a sample size with which to form generalizations about human effects to low-dose radiation and the exposure of the survivors to other health hazards have the potential to result in the same health effects. The BEIR VII report concludes that while it is unlikely that there is a threshold of exposure to low-dose radiation below which cancers are not induced, the number of radiation-induced cancers will be small at low radiation doses (BEIR VII, 2006). While moderate radiation doses cause well-documented, non-stochastic (acute) effects, one cannot measure significant effects at low-level radiation dose.

However, one of the problems that arises in studying low-level radiation is defining low-level radiation (Brues et al.1959). One way of clarifying the idea of low-level radiation exposure is by establishing how any radiation exposure, regardless of how the exposure is received over a period of years, is low-level if the effects of the exposure require many years to become apparent (Brues et al.1959). The BEIR VII report defines low-level radiation doses from zero up to about 100 milliSievert (0.1 Sievert) of low-LET radiation (BEIR VII, 2006). The Sievert is a unit of radiation that is a combination of the absorbed dose deposited within the tissue and the type of radiation providing the energy (i.e. gamma, beta, or alpha). The BEIR VII report also provides

conclusions regarding the humans exposed to radiation during the incident in Chernobyl.

There were multiple communities near the Chernobyl nuclear accident exposed to low-levels of radiation. In Chernobyl, the estimated incidence of radiation-induced cancer rose by 3% in the affected areas while there was a 99% survival rate for the thousands of children who contracted thyroid cancer with the implication that poverty and stress posed a much greater threat to the local communities than radiation fallout (Valeska, 2005). The increased incidence of thyroid cancer was radiation dose dependent within the population around the Chernobyl areas (BEIR VII, 2006).

Psychological Effects of Radiation Exposure

The Chernobyl nuclear power plant explosion on April 26, 1986 was one of the largest releases of radioactive material from a reactor accident in the twentieth century (Adams et al. 2011). Radioactivity from the high-yield fission products Mo-99, Ru-103&106, I-131&132, Te-132, Cs-134&136&137, and Ba-140 was detected by many countries in the northern hemisphere in the first two weeks of May 1986 due to the fallout from the Chernobyl Nuclear Power Plant (Chung et al. 1986). While the long-term human physical health impact and cancer risk was not as drastic as was originally expected at the time of the incident, the long-term mental health consequences have been great (Adams et al. 2011). Poorer psychological well-being among the evacuees (twenty years after the accident) due to the continued non-resolvable concerns about the physical health risks from the accident has also been an ongoing consequence

(Adams et al. 2011). While there was a dramatic increase in thyroid cancer among exposed children ten years after the Chernobyl accident, there were no other serious health effects observed with the exception of the long-term mental health effects (Havenaar et al. 1997).

In addition to the higher levels of psychological distress in the affected population, there was a high prevalence of DSM-III-R disorders in the severely affected Gomel region in Belarus, with a significantly higher risk among evacuees and mothers with young children (Havenaar et al. 1997). More evacuee teens from areas of Chernobyl reported negative risk perceptions than the controls from other areas had reported (Bromet et al. 2011). Additionally, there is mounting evidence that many lifetime psychiatric disorders will first appear in childhood or adolescence (Costello et al. 2006). The children who are exposed to such higher levels of psychological distress following a disaster are more likely to succumb to a psychiatric disorder that will affect them in their adult lives. The most common mental health consequences of disasters are depression, anxiety, post-traumatic stress disorder, medically unexplained somatic symptoms, and stigma (Bromet, 2012). Unfortunately, the long-term psychological effects of disasters have not been well studied and more research is necessary (Boice, 2012).

Disasters involving radiation are particularly pernicious because the exposure is invisible and universally dreaded, and the consistent psychological distress of the exposure can pose a long-term threat to health (Bromet, 2012). After the Chernobyl

disaster, studies of clean-up liquidator workers and adults from contaminated areas found a two-fold increase in post-traumatic stress (along with other mood and anxiety disorders) and significantly poorer subjective ratings of health (Bromet, 2012). While the most important risk factor was severity of exposure among clean-up workers, the major risk factor in the general population was perceived exposure to harmful levels of radiation (Bromet, 2012). Due to the comorbidity of mental health and physical illness, the higher rates of mental health issues within a population exposed to a radiation disaster are a concern and must be further examined to eventually reduce the impact of the psychological distress. Survivors of the atomic bombings in Hiroshima and Nagasaki also reported higher rates of anxiety and physical illnesses not specifically related to any disease twenty years after the 1945 bombings (Boice, 2012).

Since mental health is a leading cause of disability, physical morbidity, and mortality, health monitoring after radiation accidents like Fukushima should include standard measures of well-being (Bromet, 2012). Unfortunately, due to a common Japanese stigma towards individuals who admit to having mental health issues, there is a great deal of difficulty in addressing the psychological distress caused by the Fukushima incident and the subsequent mandatory evacuation. The Fukushima residents affected by the nuclear power plant disaster have been reticent in obtaining the mental health services they need to reduce their fears regarding the radiation contamination from the incident and alleviate the stress from their lifestyle changes after moving from their homes.

The common fear within the general public regarding any radiation incident has also greatly affected the outcome of governmental efforts in Fukushima, Japan. One of the many problems that make the evaluation of cancer and non-cancer disease risk in Fukushima difficult is the lack of trust of the Fukushima residents due to the common theory that the Japanese government and local authorities are hiding important information (Akiba, 2012). The estimated doses to workers and to the public in Fukushima are too small and have too many sources of confounding bias (i.e. Hepatitis C virus, cigarette smoking, medical x-rays, CT/nuclear medicine imaging, etc.) that result in the same health effects as radiation exposure and are interfering in the creation of a study that would result in a detectable increase in cancer (Boice, 2012). Extensive screening of the Fukushima residents with diagnostic radiation procedures for discovering incidents of cancer should not be performed as the extensive medical screening can result in more radiation exposure than from the Fukushima accident (Boice, 2012).

Fukushima Daiichi Nuclear Power Plant Disaster

An earthquake registering 9.0 on the Richter scale occurred at 2:46 p.m. on March 11, 2011 off the Pacific coast of Japan and hit the northeast of Japan (Hazama et al. 2013). The three operating reactors at Fukushima Daiichi nuclear power plant shut down automatically (Hazama et al. 2013). The earthquake was followed by several tsunamis (Hazama et al. 2013) and a large tsunami 41 minutes after the earthquake provided a massive wall of rolling water that flooded the emergency generators, leaving the plant without power for the cooling systems for the plant cores (Hazama et al. 2013).

The three reactors melted down several days later (Unit 1 at 3:36 p.m. on March 12, Unit 3 at 11:01 a.m. on March 14, and Unit 2 at 6 a.m. on March 15) due to lack of electrical power to cool the radioactive cores (Ishikawa, et al. 2012). The reactor meltdown induced the release of hydrogen gas, causing explosions in the reactor buildings, and the release of I-131, Cs-134, and Cs-137 into the atmosphere (Ishikawa, et al. 2012). Due to potential concerns of human over-exposure to radioactive contamination, all residents within a 3 km radius of the Fukushima Daiichi power plant were ordered to evacuate on March 11, 2011, and residents within a 20 km radius were ordered to evacuate on March 12, 2011 (Evacuation Orders and Restricted Areas, 2017).

Radionuclides in Environment after Nuclear Accidents

After the Fukushima Daiichi reactors meltdown and subsequent explosions, the contaminated tsunami water within the reactors were released into the ocean to dilute the concentration of the radionuclides and remove the contaminated water from the reactor area to avoid further contamination of humans. Although some radionuclides were significantly elevated in the ocean water near the discharge point of the Fukushima-Daiichi nuclear power plant after the nuclear accident, dose calculations suggest minimal impact on marine biota or humans (Buesseler et al. 2011). The minimal radioactive impact from direct exposure in the surrounding ocean waters is due to the lack of humans and marine biota in the immediate vicinity of the discharge point and the dilution of the contaminated water within the ocean body (Buesseler et al. 2011).

In order to properly model the radiation doses received in an area with long-term radiation exposure, it is necessary to evaluate which contributions to dose are important and which are not (Anderson, 2006). The external radiation dose of the animal species in question needs to include approximate doses of the types of vegetation the animal is externally exposed to, the amount of radiation available within the specific vegetation, and the radiation activity levels in soil. The trees, grasses, shrubs, etc. the animal is externally exposed to can provide a radiation dose to the animal if the radionuclides present on the surfaces of the vegetation emit beta or gamma energies to the animal as the animal walks by the vegetation or by depositing radionuclides on the surface of the animal during contact. The external doses also need to include the changing external environment and the transport of the radionuclide(s) providing the radiation dose. The consistently changing ecological systems in the areas of long-term radiation contamination have a considerable effect on the radionuclides of interest through the amount of dose from the nuclide and the availability of the nuclide in transferring from one area to another. The significance of ecological processes was seen from the fact that contamination of agricultural products in Austria was decreasing continuously since the Chernobyl accident, whereas the Cs-137 activity measured in plants and wild animals in the forest was still elevated (Bossew et al. 2001). A similar effect was seen in Fukushima where the preliminary estimated dietary dose levels among Fukushima residents were much lower than the maximum permissible dose 1 mSv/year, based on new Japanese standard limits for radiocesium in foods (100 Bq/kg for general foods) (Harada et al. 2013).

While Cs-137 becomes trapped in certain kaolinite clay soils and will not easily transfer out of the soil, the radionuclide has a great affinity for water and can transfer from one water-based plant or organism to another. The measurement of Cs-137 in plants can give an estimate of radiation available in the environment. The radioactivity of various terrestrial and aquatic vegetation characteristic of Mediterranean countries was measured after the Chernobyl accident as lichens and seaweeds are considered as bioindicators of radioactive contamination do to the ability of the plants to uptake and store radioactive nuclides such as Cs-137 (Barci et al. 1988). The concentration of long-lived fission nuclides remaining three months after the Chernobyl accident was found to be enhanced in leaves with a needle shape and in lichens (Barci et al. 1988). However, differing plant species have differing affinities for differing radionuclides. For example, the seaweed Sphaerococcus exhibits a strong specific activity for iodine and ruthenium elements and poor concentration for cesium nuclides (Barci et al. 1988).

Research of the radioactive contamination after the Chernobyl accident also indicated a differing radionuclide deposition within different animal species. Cs-137 and K-40 activities were determined by the gamma-spectrometric method in 49 meat samples of five large game species (brown bear, wild boar, roe deer, red deer, and chamois) in the mountain forest region of Gorski Kotar in Croatia approximately 25 years after the Chernobyl accident (Sprem et al. 2013). The results indicated that the roe deer, red deer, and chamois (herbivore game species) showed significantly lower cesium concentrations than the brown bear and the wild boar (omnivore species) indicating that different dietary methods impacted cesium concentrations in meat

(Sprem et al. 2013). Analysis of the estimated effective dose equivalent from each of the large game species showed that the uptake of the highest cesium doses was from the consumption of omnivore species meat, while much lower doses was incorporated with the consumption of meat from herbivore species (Sprem et al. 2013). The components of and the state of the ecosystem within the radiological release area must be thoroughly analyzed in terms of radionuclide transport in order to obtain a more complete understanding of the potential sources of external radiation dose for any human and/or animal species of interest so the possible extent of the types of radiation damage can be evaluated.

Ionizing Radiation Effects

Ionizing radiation can cause biological changes within a living system by interacting with multiple targets at the cellular level, such as DNA. Radiation levels of 0.100 Gy can cause the onset of deterministic effects in humans. Higher levels of ionizing radiation (upwards of 1 Gy) can overcome the human body's natural healing protocols and result in specific deterministic effects such as cataracts, erythema, nausea, vomiting, and diarrhea (acute radiation sickness). The deterministic effects of radiation exposure to humans occur immediately and are a result from a specific range of higher doses of exposure and have specific radiation dose thresholds which have been accepted by the scientific community. The stochastic effects of radiation exposure are long term effects such as cancer and can result from low doses of radiation exposure.

The stochastic effects of radiation occur many years after radiation exposure and are caused by DNA damage. Ionizing radiation can directly cause double strand breaks (DSBs) or single strand breaks (SSBs) in the DNA chain or create hydroxyl water molecules that damage DNA strands. Differing types of radiation generate distinct classes of DSBs, interact with cellular repair proteins in different ways, and the repair kinetics of high-linear energy transfer (LET) radiation induced DSBs are different from those created by low-LET gamma radiation (Bekker-Jensen, 2006).

Low-LET radiation includes radiation from beta particles and gamma rays while high-LET radiation is from alpha particles and neutrons. Alpha particles are considered to have a somewhat higher efficiency than the other radiations (beta or gamma) in producing many types of biological damage (Brues, et al. 1959). Alpha particles are much larger and heavier than beta particles or gamma rays and result in greater damage to DNA during impact. While alpha particles travel short distances, cannot penetrate skin, and are most hazardous when ingested, gamma rays and beta particles can penetrate skin to damage DNA within an organism. DNA damage is related to detrimental health effects, but the extent and type of the health effects are not clear for low dose radiation. There are many challenges associated with understanding the health effects of low doses of low-LET radiation (BEIR VII, 2006).

Disagreements about the stochastic effects from low doses of ionizing radiation stem from two problems with the interpretation of observational data (Land, 1980). The precise direct estimation of small risks requires impracticably large samples (Land,

1980). Also, the precise estimates of low-dose risks based largely on high-dose data, (where the sample size requirements are easily satisfied) must depend heavily on assumptions about the shape of the dose-response curve (Land, 1980). The sample size requirements are easily satisfied for high radiation dose risk estimates because higher cancer risks are associated with higher radiation dose and smaller samples sizes are needed for higher statistical power. As the radiation risk decreases due to a lower radiation dose, a larger sample size is necessary in order to detect the resulting radiation effect (Land, 1980). Due to the lack of data on biological effects from low-level radiation exposure and the many other variables involved in producing the same health effects, there has been considerable debate over the existence of low-level threshold radiation doses resulting in future illnesses. Due to the inability to determine the existence of a threshold for stochastic effects of radiation, the LNT model is utilized in order to protect radiation workers and the public against any long-term health effects of radiation.

There are many compounding variables that can affect an individuals' state of health when exposed to an outside stressor that causes biological damage such as radiation. As individuals age, the older organs within the individual have a diminished capacity to cope with diverse acute and chronic stresses (Jazwinski, 1996). Potential confounding between radiation dose and the severity of cancerous disease may explain some of the increased cancer risk observed (Doody et al. 2000). The healing capabilities of humans also vary across the population and can be markedly different than that of other species due to different environmental and man-made stressors such

as radiation. The healing capabilities of humans and animals are controlled by the genes within DNA. The diverse genes involved in gene silencing, DNA repair, genomic stability, and growth factor signaling are strong determinants of life span in a variety of species (Dorman et al., 1995; Guarente, 1996; Wright et al., 1996; Smeal and Guarente, 1997) (Rudolph et al. 1999). The analysis of the deviations of these genes in DNA exposed to radiation from the normal appearance can be used as a surrogate for radiation exposure and is a field known as biodosimetry.

Biomarkers of Ionizing Radiation

Biodosimetry is the estimation of received doses by determining the frequency of radiation-induced chromosomal aberrations and is widely applied in humans acutely exposed to radiation as a result of accidents or for clinical purposes (Ulsh et al. 2003). The technique of biodosimetry in humans can be utilized to determine an estimated radiation dose within three days of a radiological event. In biodosimetry, no assumptions are required regarding external exposure rates and the movement of organisms into and out of contaminated areas (Ulsh et al. 2003). Biodosimetry provides a genetically relevant biomarker of cumulative lifetime radiation exposure (Ulsh et al. 2003). Specific biodosimetric techniques can be performed to determine if an organism experienced significant chromosomal changes from a single or prolonged radioactive exposure.

At the present time, chromosome aberrations observed in the peripheral blood lymphocytes of an individual may be used in biodosimetry as an indicator of radiation exposure (Hall et al. 2003). Chromosomal aberrations such as dicentric chromosomes

and acentric fragments are a result from a break in two chromosomes in the DNA of a cell during interphase and the rejoining of the DNA into a distorted chromosome and fragments (Hall et al. 2003). The dicentric rejoining eventually leads to the reproductive death of the cell (Hall et al. 2003). However, research has shown the prolonged existence of dicentrics chromosomes in cancer cells (MacKinnon et al. 2011) and the persistence of dicentric chromosomal aberrations in atomic bomb survivors (Awa et al. 1978). The stability of dicentrics also depends on the organism (Stimpson et al. 2012). The lymphocytes in a blood sample can be cultured; forced to divide via mitogens; have cell division arrested; and have the resulting chromosomes viewed via microscope so that the dicentrics and acentrics can be scored (Hall et al. 2003). The number of dicentrics observed from a blood sample of an individual exposed to an unknown amount of radiation can be compared to in-vitro cultures exposed to known doses (dose response curve) and an estimated dose can be obtained for the unknown amount of radiation exposure (Hall et al. 2003).

A dose response curve is a graph of observed chromosomal aberrations versus specific radiation doses. While there are multiple variations within dicentric dose response curves for humans due to confounding variables such as genetics, other environmental toxicities, diet, etc., and type or duration of radiation exposure, a study creating a dicentric dose response curve for human blood irradiated with 1 Gy dose of 250 kVP x-rays found 304 dicentric and ring chromosomes for a total of 2,800 cells that were analyzed (Pajic et al. 2014). The dose response curve forms a linear quadratic

relationship when the two chromosome breaks are a result of two particle hits (Catchside et al. 1946).

Using a similar technique, the existence of micronuclei, a small chromosomal fragment that was not incorporated into a dividing cell during cell division, can be observed and can also be used to estimate radiation dose via an established dose response curve. The micronucleus technique is a method for measurement of chromosomal damage in mitogen-stimulated human lymphocytes (Fenech et al. 1985). Micronuclei require one cell division to be expressed (Fenech et al. 1985). For human lymphocytes irradiated in vitro, there is a linear relationship between dose of radiation and number of induced micronuclei which was observed due to the success of quantifying micronuclei in lymphocytes via the cytokinesis-block method (Fenech et al. 1985).

Since the number of dicentrics/acentrics and micronuclei decline as time progresses, dicentrics and micronuclei are known as “unstable” chromosomal aberrations and radiation biomarkers of effect (Hall et al. 2003). Dicentrics and micronuclei will eventually provide an underestimation of dose as time progresses after radiation exposure (Hall et al. 2003). Translocations are chromosomal aberrations that are a result of chromosomal breaks and subsequent rearrangement of non-homologous chromosomes and have the ability to continue to exist after subsequent cell divisions (Hall et al. 2003). As translocations continue to exist for many years after radiation exposure, translocations are considered to be “stable” chromosomal aberrations and

are considered a radiation biomarker of exposure which can be used to provide a more accurate dose estimate years after radiation exposure (Hall et al. 2003).

The fact that many of the radiation exposed cells can survive in the body of an irradiated human for twenty and more years is now well established (Buckton et al. 1978). What correlation may exist between the irradiation-damaged cells and possible late effects of the irradiation, such as the increased frequency of mid-line cancers and leukemia, still remains an enigma (Buckton et al. 1978). For example, when a sister chromatid exchange (SCE) occurs as the breakage of four strands of DNA, a switch of the strands from one to the other arm of the same chromosome, and the rejoining of those strands in their new location, the question that remains to be answered is whether the breakage and rejoining occurs without producing any modifications in the genetic code (Carrano, 1986). Further research on the sites of radiation-induced chromosome exchange in cells that can survive in-vivo, on the chromosome sites involved in cells that are observed to form clones, and on the cytogenetics of radiation-induced malignancies, can reveal patterns of chromosomal changes that can be interpreted as either harmless or as the commencement of dangerous malignancies (Buckton et al. 1978). The analysis and subsequent correlation of chromosomal aberrations with radiation dose is a bio-dosimetric technique that can one day be used to reveal the incidence of malignant health concerns from radiation exposure.

The analysis of dicentric chromosomes in human peripheral blood lymphocytes by Giemsa staining is the most established method for biological dosimetry (Shi et al.

2012). However, the dicentric analysis via the Giemsa staining method requires a well-trained investigator due to the difficulty in detecting aberrations quickly and accurately (Shi et al. 2012). Additionally, the Giemsa staining method is very time consuming due to the high number of chromosomal metaphases that need to be analyzed in order to obtain statistically relevant results. For example, at low radiation doses, only 15 dicentrics were found per 2800 human lymphocyte metaphase spreads analyzed (Pajic et al. 2014). Another technique for analyzing dicentric aberrations in metaphase chromosome spreads is via FISH (Fluorescence In-Situ Hybridization). Although accuracy in detection of chromosome abnormalities by FISH techniques is higher than that by Giemsa analysis, the acquisition of consistent results with multi-color FISH analysis requires high-quality FISH techniques which require expensive fluorescent dye creations (Shi et al. 2012). The analysis of translocations within the chromosome of human blood exposed to low-LET gamma rays indicates a higher frequency of translocations in comparison to dicentrics due to limitation of the number of centromeres for the dicentrics, whereas there is no such limitation for the induction of translocations (Matsumoto et al. 1998).

Analysis of chromosomal aberrations was performed on blood lymphocytes of human blood exposed to Co-60 gamma rays and dose response curves were created for dicentric aberrations seen via Giemsa staining and FISH painting (Lindholm et al. 1998). Differences in chromosomal aberrations scoring criteria resulted in large uncertainties surrounding the linear component of the dose response at the low doses (Lindholm et al. 1998). Dose reconstruction of past radiation exposures for humans in

cases of low doses is very dependent on the linear coefficient of the equation fitting the control dose-response curve (Lindholm et al. 1998). Due to the lack of observable chromosomal aberrations from low levels of radiation, the dose-response curve section at the low radiation doses is inconsistent and can affect the dose estimate ascertained. The dose-response curve for higher levels of radiation is well established. Other radiation dosimetry analysis techniques that have been used for higher radiation activity samples include liquid-scintillation counting, inductively coupled plasma mass spectrometry (ICP-MS), laser resonance ionization mass spectrometry (RIMS), and surface ionization mass spectrometry (SIMS) (Straume et al. 2006). The biodosimetry technique of chromosomal aberration analysis has been used extensively for low dose radiation exposure.

The biodosimetry technique of measuring chromosomal abnormalities to estimate radiation dose has been used in studies involving radiation workers. A FISH method was used to measure chromosome aberration rates in lymphocytes of 30 retired plutonium workers with combined internal and external radiation doses greater than 0.5 Sv along with 17 additional workers exposed to hazardous substances during the course of employment (Livingston et al. 2006). Radiation exposures to the plutonium worker group were primarily the result of internal depositions of plutonium and plutonium's radioactive decay products resulting from various work-related activities and accidents (Livingston et al. 2006). The study discovered that elevated rates of stable chromosome aberrations were found in lymphocytes of former workers decades after plutonium intakes, providing evidence that chronic irradiation of bone marrow stem cells

induces cytogenetically altered cells that persist in peripheral blood (Livingston et al. 2006). While research involving radiation exposure to humans has provided consistent results with damaged DNA, radiation research on other wildlife forms has provided differing conclusions.

When Fuma et al. in 2010 conducted radiation exposure research on aquatic microbial populations, the population decrease effects were not dependent on radiation doses, as some microbial populations in the irradiated microcosm were larger than those of the control (Fuma et al. 2010). The unexpected results were regarded as indirect effects through interspecies interactions from population changes in other organisms co-existing in the irradiated microcosm (Fuma et al. 2010). The conclusion was that some indirect effects on consumers and decomposers likely arose from inter-species competition within each trophic level and that prey-predator relationships between producers and consumers caused some indirect effects on producers (Fuma et al. 2010). Another extensive radioecological study conducted by Zaitsev et al. in 2013 around Chernobyl discovered that despite the high resistance of most of the soil-dwelling organisms (Tardigrada, Nematodes, millipedes, collembolans, and oribatid mites) to ionizing radiation, some soil animals (earthworms) were very vulnerable to radioactive contamination due to low motility, direct contact with hot particles, and radioisotope accumulation in soil. Other experiments using extremely high doses of radiation (from Pu-239 up to 50 Gy) did not produce a lower population response among amoebae and the same effect was observed for other protists at higher doses at 500 Gy (Krivolutsky et al., 1988). In an ecosystem exposed to a new or foreign influence

that can potentially affect multiple species within the environment, the analysis of the interaction and effects of the relationships the species have with the surroundings are vital in order to determine the future effects of the foreign influence on the species in question.

Wild Boar Information

Wild boar are an animal species that are considered to be very intricately involved within the ecosystem and are very similar biologically to humans which make them useful as a model species for study (Kobayashi et al. 2012). Wild boar are a sentinel species to humans due to morphological and metabolic similarities. The transplantation of pig organs into humans are currently being investigated as a solution for the increasing shortage of human donor organs (Ekser et al. 2009). After the disaster at Fukushima Daiichi and the subsequent evacuation of the humans in the local area, the Japanese wild boar have increased in population and have invaded the vacant towns in the prefecture (Tanoi, 2016). As there are no humans living within the areas of the Fukushima prefecture that still have annual radiation doses above 50 mSv, a human study investigating the effects of low-level, lingering radiation at Fukushima cannot be accomplished and a suitable animal model is necessary. One of the merits of biological monitoring is that the condition of a resident population of plants or animals can be used to survey improvement or deterioration of the environment at a specific site, or to compare resident populations with those at reference sites (Loar et al. 1992). A detailed study involving Japanese wild boar living within the exclusion zone in the Fukushima prefecture can not only indicate the effects of low-level, persistent radiation, but can

also provide information regarding the overall state of the ecosystems with the encompassing areas.

Unfortunately, while the physical and morphological aspects of most wild boar species and sub-species have been thoroughly investigated, there are little data on the Japanese wild boar (Sus scrofa leucomystax). Many wild boar species have been known to traverse territories spanning several miles, but published data on the territory size of the Japanese wild boar are not yet available. Wild boar are omnivores and are known to burrow through topsoil in order to obtain plants and to hunt in order to eat small rodents. The wild boar top soil burrowing is a trait that exposes the Japanese wild boar living within the Fukushima prefecture to available radionuclides (Cs-134 and Cs-137) in the ground.

The internal digestion and retention of radionuclides by the wild boar can affect the wild boar DNA within the blood. Wild boar generally have 36 chromosomes (18 chromosome pairs) while some species of wild boar can have 37 chromosomes (Silva et al. 2011). There are current investigations underway that are performing detailed chromosomal karyotyping of the Japanese wild boar as there have not been any previous investigations on Japanese wild boar chromosomes. There are no published data on analyzing any wild boar blood exposed to radiation for chromosomal aberrations, irradiating wild boar blood, or the specific techniques to process wild boar blood for chromosomal analysis. One study involving Japanese wild boar was conducted by Tanoi et al. in 2016.

The concentrations of radioactive cesium in different organs of wild boar inhabiting the town of Iitate, Fukushima were measured after the Fukushima Daiichi nuclear power plant accident by collecting 24 wild boar samples and measuring radiation concentration using a NaI gamma ray counter in 2012 (Tanoi et al. 2016). The radiocesium concentration (Cs-134 and Cs-137) in the wild boar samples was highest in muscle (approximately 15,000 Bq/kg) and low in ovary, bone and thyroid gland, indicating a large variation among tissues within the wild boar (Tanoi et al. 2016). The wild boar in the Fukushima prefecture of Japan will have higher radiocesium muscle concentrations if the wild boar have habitats that have not been decontaminated from radionuclides (Anderson et al. 2017). The variation of muscle wild boar radiocesium concentrations is a combination of environmental exposure to radiation and the ability of the wild boar to internally processes and remove radiocesium. Different components of the Japanese wild boar collect varying levels of radioactive cesium which can affect the internal radiation dose the wild boar receives and subsequently affect the chromosomal aberrations.

Pilot Study Goals

The goal of the study was to determine if blood biomarkers in wild boar were suitable for determining radiation dose.

The objectives of the pilot study were as follows:

1. Collect wild boar blood and ascertain if a dose response relationship is present in wild boar

2. Creation of wild boar dose response curve from control data
3. Compare estimated wild boar dose with acentric/dicentric measurement

For the Quantifying Biomarkers in Wildlife Exposed to Low Doses of Environmental Radiation pilot study, control wild boar blood was collected from Kentucky, USA and irradiated at Colorado State University. The control wild boar blood was obtained by USDA hunters in Kentucky from six wild boar euthanized for research purposes and shipped overnight to Colorado State University. The six control wild boar blood samples were then irradiated at varying low doses of radiation.

The irradiated wild boar blood was processed via human cell culture chromosome stimulation protocols to initially determine if the blood cell processing protocols would be effective in ensuring cell progression through the cellular cycle, through the creation of metaphase cellular spreads, and through cell division. When the blood processing protocols were effectively utilized, a dose response relationship was created via dicentric analysis within metaphase chromosomal spreads in the control wild boar blood exposed to gamma ray radiation dose. Another dose response relationship was also created via micronuclei analysis within bi-nucleated cells in the control wild boar blood exposed to gamma ray radiation dose.

Wild boar blood from areas of the Fukushima Prefecture in Japan was collected, processed via the blood cell processing protocols that were determined to be effective in obtaining metaphase spreads and bi-nucleated cells, and analyzed for chromosomal

aberrations. The chromosomal aberrations were compared with the chromosomal aberrations observed in the irradiated control wild boar blood in order to verify the feasibility of the biodosimetry technique and to potentially estimate the radiation dose to the Japanese wild boar in Fukushima.

The hypothesis of the pilot study was that it is possible to estimate chronic radiation dose to wild boar exposed to low levels of lingering environmental ionizing radiation in Fukushima prefecture as well as in irradiated blood from wild boar residing in areas experiencing only natural background radiation with biodosimetry techniques.

MATERIALS AND METHODS

Institutional Animal Care and Use Committee (IUCAC) Exemption

The quantifying biomarkers in wildlife exposed to low doses of environmental radiation pilot study has an animal use authorization[1].

Obtaining Wild Boar Blood Samples from Fukushima Prefecture, Japan

Wild boar blood was obtained inside the Fukushima Prefecture via assistance of professional Japanese boar hunters. Japanese boar hunters lay traps to capture Japanese Wild Boar for governmental purposes of culling the nuisance population. The traps are checked every morning by the Japanese boar hunters. The hunters dispatched the Wild Boar either through electrical shock or rifle bullet through the head. Researchers from the International Institute of Environmental Radioactivity (IER) at Fukushima University, Japan are then contacted to collect the wild boar blood. The wild boar blood was collected through a vein in the neck of the boar via a syringe and an 18 gauge needle and placed into a heparinized tube for transport to the microbiology lab at the IER. The blood was then processed either for dicentric analysis via creation of metaphase spreads or micronuclei analysis via creation of bi-nucleated cells, dropped onto slides, sealed with DPX mounting media and coverslip, and prepped for shipment to Colorado State University with the approved USDA United Stated Veterinary Permit for Importation and Transportation of Controlled Materials and Organisms and Vectors Number 132546 (see Appendix H).

[1] The IACUC (Institutional Animal Care and Use Committee) of Colorado State University approved the pilot study on May 5, 2016 (See Appendix A).

Obtaining and Irradiating Control Wild Boar Blood Samples from Kentucky, USA

Wild boar blood (as a control) was donated by USDA hunters from Kentucky, USA. The Wild Boar Blood was obtained immediately post-mortem. The control blood was obtained with syringes via a vein in the neck and stored at room temperature in heparinized vials from six wild boar. After overnight shipment to Colorado State University (CSU) via FedEx, the blood was separated into 7 vials per wild boar (42 vials total) and irradiated at the irradiator in Room 4 at the Molecular and Radiological Health Sciences Building at CSU to 0 Gy (as control), 0.1 Gy, 0.2 Gy, 0.3 Gy, 0.5 Gy, 1 Gy and 2 Gy. The blood was irradiated for the times depicted in Table 1 by Cs-137 gamma rays at a dose rate of about 6 Gy/hr inside the irradiator on a table 41.75 cm from the source (Marcinko et al. 2017).

Table 1: Control Wild Boar Blood Irradiation Times and Doses

Control Blood Time in Irradiator	Dose to Control Blood
0 minutes	0 Gray
1 minutes	0.1 Gray
2 minutes	0.2 Gray
3 minutes	0.3 Gray
5 minutes	0.5 Gray
10 minutes	1 Gray
20 minutes	2 Gray

The irradiated blood was then processed for dicentric analysis via creation of metaphase spreads or micronuclei analysis via creation of bi-nucleated cells at Dr.

Susan Bailey's Biological Safety Cabinet 1 Laboratory at the Molecular and Radiological Health Sciences building at Colorado State University.

Metaphase Spread Analysis for Dicentrics (see Appendix B, C, D, and G)

The wild boar blood was cultured in RPMI-1640 medium supplemented with 1% L-glutamine, 30% FBS and 1% antibiotic-anti-mycotic at 37°C for 68 hours. The following primary antibodies were used to induce cell division: 50 µg/mL PMA and 1 µg/mL Ionomycin. At 68 hours, 0.1 µg/mL of Colcemid was added to the cultures to stop cell division at metaphase and the cultures were placed back into the incubator. At 72 hours, the cells were washed with 3:1 methanol:acetic acid solution and treated with a hypotonic solution (75mM KCl) for 30 min at 23°C. The cells were washed and centrifuged five times with 3:1 methanol: acetic acid fixative to remove all the red blood cells from the lymphocytes. Cells were dropped onto slides wet with cold, deionized-water and stored in a humidity chamber for 24 hours to age. After 24 hours, slides were immersed in 5% Giemsa stain in Gurr buffer solution for 8 min, rinsed in deionized water for 10 minutes, and allowed to dry. Coverslips were placed onto slides via DPX Mounting Serum. For the Kentucky Wild Boar control samples, 100 metaphase spreads per sample/dose were analyzed for all observable dicentrics under a bright field microscope at 1000x magnification. For Fukushima wild boar blood samples, 200 metaphase spreads per sample/dose were analyzed under a bright field microscope at 1000x magnification.

Bi-nucleated Cell Analysis for Micronuclei (see Appendix B, E, F, and G)

The wild boar blood was cultured in RPMI-1640 medium supplemented with 1% L-glutamine, 30% FBS and 1% antibiotic-anti-mycotic at 37°C for 44 hours. The following primary antibodies were used to induce cell division: 50 µg/ml PMA and 1 µg/mL Ionomycin. At 44 hours, 3 µg/mL of Cytochalasin B was added to the cultures to stop cell division and the cultures were placed back into the incubator. At 72 hours, the cells were washed with 3:1 methanol:acetic acid solution. The cells were washed and centrifuged four times with 3:1 methanol: acetic acid fixative to remove all the red blood cells from the lymphocytes. Cells were dropped onto slides wet with deionized-water and stored at -20°C until ready for Giemsa staining. For Giemsa staining, slides were immersed in 5% Giemsa stain in Gurr buffer solution for 8 min, rinsed in deionized water for 10 minutes, and allowed to dry. Coverslips were placed onto slides via DPX Mounting Serum. For Kentucky Wild Boar control samples, 100 bi-nucleated cells per sample/dose were analyzed for all observable micronuclei under a bright field microscope at 1000x magnification. For Fukushima wild boar blood samples, 200 bi-nucleated cells per sample/dose were analyzed for all observable micronuclei under a bright field microscope at 1000x magnification.

RESULTS

Control Wild Boar Blood Dicentrics

In Table 2, the number of dicentrics observed in control wild boar blood irradiated with radiation were depicted. Each control wild boar blood sample was assigned a random sample number prior to dicentrics analysis in accordance with the IAEA biodosimetric sampling strategy (Dosimetry, 2011).

Table 2: Dicentrics in Control Kentucky Wild Boar Irradiated Blood Samples

Wild Boar Number	Radiation Dose Received (Gy)	Random Sample Number	Metaphase Spreads Analyzed	Dicentrics Found
1	0	15	0	N/A
1	0.1	32	0	N/A
1	0.2	82	0	N/A
1	0.3	13	0	N/A
1	0.5	83	0	N/A
1	1	62	0	N/A
1	2	46	0	N/A
2	0	20	100	0
2	0.1	68	100	0
2	0.2	79	100	0
2	0.3	70	100	0
2	0.5	4	100	0
2	1	10	100	1
2	2	52	100	0
3	0	97	100	0
3	0.1	29	100	0
3	0.3	92	20	0

3	1	26	100	1
4	0	55	60	0
4	0.2	36	100	0
4	0.5	93	100	0
4	1	12	0	0
4	2	63	100	1
5	0	37	100	0
5	0.1	30	100	0
5	0.2	41	100	0
5	0.3	35	100	1
5	0.5	53	100	0
5	1	58	100	0
5	2	99	100	1
6	0	86	100	0
6	0.1	90	100	0
6	0.2	25	100	0
6	0.3	49	100	0
6	0.5	73	100	0
6	1	51	100	0
6	2	1	100	3

In Figure 1, the number of dicentrics per metaphase spread were graphed versus the radiation dose to each of the control wild boar blood sample.

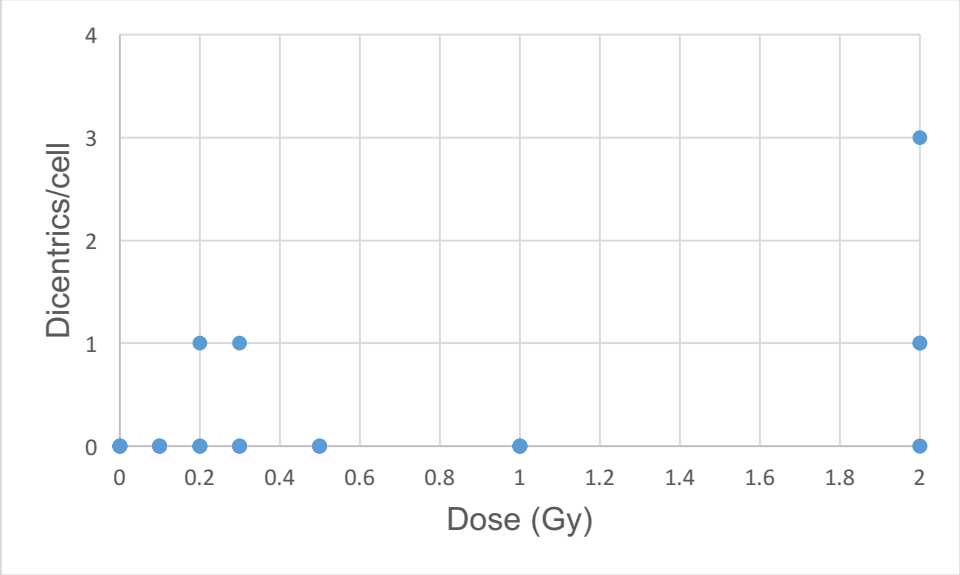

Figure 1: Dicentric Dose Response Curve for Control Kentucky Wild Boar Irradiated Blood

Figure 2 depicts a normal metaphase spread without any chromosomal aberrations from a control wild boar blood sample that was processed via the Metaphase chromosome processing protocol (see Appendix C).

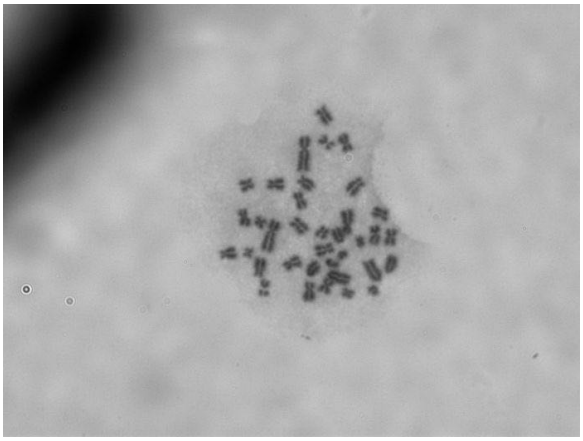

Figure 2: Control Metaphase Spread from Pig 73 (Wild Boar #6 irradiated to 0.5 Gy) without chromosomal aberrations

Control Wild Boar Blood Micronuclei

In Table 3, the number of micronuclei observed in control wild boar blood irradiated with radiation were depicted. Each control wild boar blood sample was assigned a random sample number prior to micronuclei analysis in accordance with the IAEA biodosimetric sampling strategy (Dosimetry, 2011).

Table 3: Micronuclei in Control Kentucky Wild Boar Irradiated Blood Samples

Wild Boar Number	Radiation Dose Received (Gy)	Random Sample Number	Bi-nucleated Cells Analyzed	Micronuclei Found
1	0.5	83	0	N/A
2	0.1	68	7	0
2	0.3	70	100	0
2	1	10	100	0
2	2	52	100	0
3	0	97	100	0
3	0.2	18	100	0
3	0.5	5	100	0
4	0.1	65	100	0
4	0.2	36	100	0
4	2	63	100	0
5	0	37	100	1
5	0.5	53	0	N/A
5	2	99	100	2
6	0.3	49	0	N/A
6	0.5	73	100	0
6	1	51	30	0
6	2	1	100	1

In Figure 3, the number of micronuclei per bi-nucleated cell was graphed versus the radiation dose to each of the control wild boar blood sample.

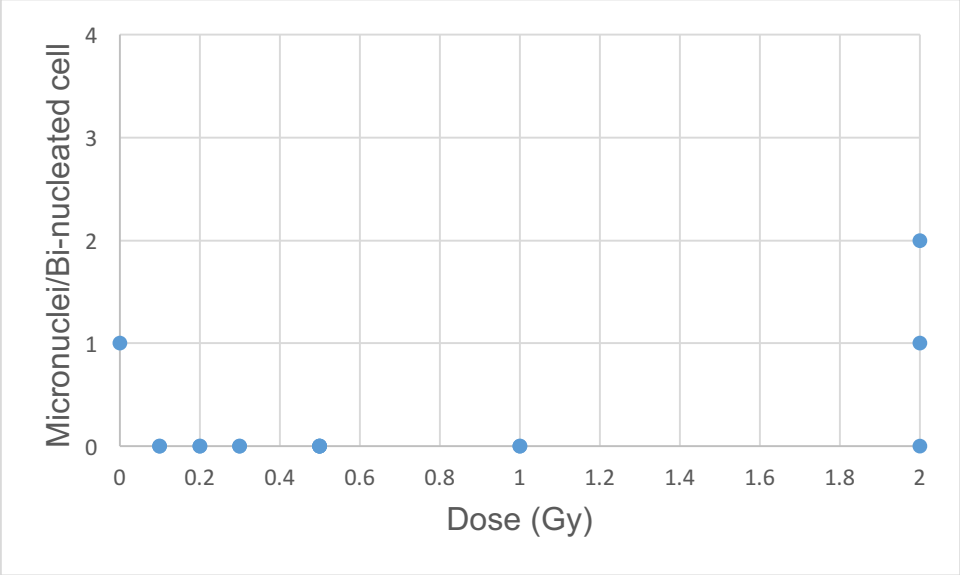

Figure 3: Micronuclei Dose Response Curve for Control Kentucky Wild Boar Irradiated Blood

In Figure 4, bi-nucleated cells from a control wild boar blood sample illustrate normal cell division without the presence of micronuclei.

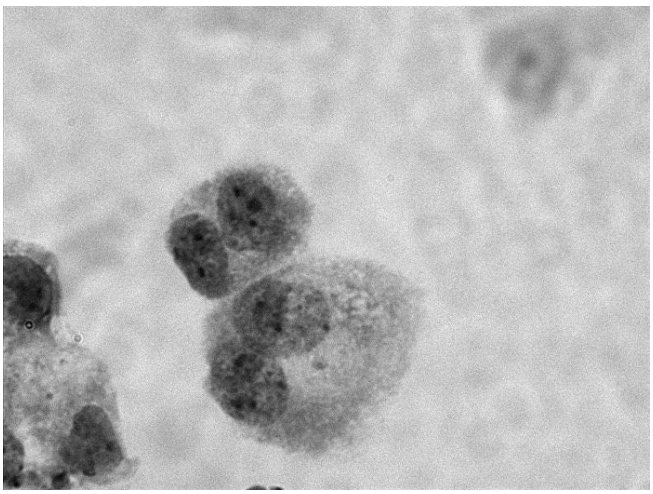

Figure 4: Control Bi-nucleated Cell from Pig 63 (Wild Boar #4 exposed to 2 Gy) without chromosomal aberrations

In Figure 5, bi-nucleated cells from a control wild boar blood sample illustrate the creation of micronuclei during cell division.

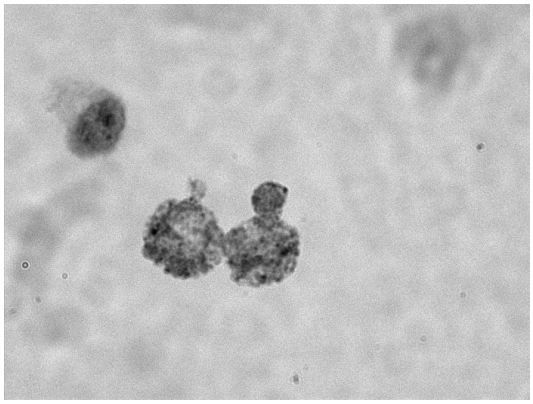

Figure 5: Control Bi-nucleated Cell from Pig 1 (Wild Boar #6 exposed to 2 Gy) with micronuclei formation

Fukushima Wild Boar Dicentric Biodosimetry

In Table 4 and Table 5, wild boar blood data are shown for samples obtained from wild boar euthanized during governmental wild boar population control procedures in the Fukushima Prefecture, Japan. The wild boar blood was obtained during daily trips into the exclusion zones (Namie, Okuma, etc) in the Fukushima prefecture in Japan and obtained by researchers from the Institute of Environmental radioactivity (IER) every morning at 1000 hrs. At 1000 hrs, the researchers from the IER would follow the governmental hunters to each of the wild boar traps. The hunters would euthanize any wild boar in the traps, and the researchers would obtain wild boar blood samples as well as other biological samples (i.e. hair, teeth, organs, tissue, etc.) for other research projects. The wild boar blood samples were then driven to the IER by 1800 hrs the same day for processing via the Metaphase chromosome processing protocol (see Appendix C) or the Bi-nucleated cell processing protocol (see Appendix E). The

processed lymphocytes would then be dropped onto slides (see Appendix D) and Giemsa stained (see Appendix G). After successful coverslip application onto the slides, the wild boar slides with metaphase spreads and bi-nucleated cells were prepared for shipment to CSU in the USA.

Two hundred metaphase chromosome spreads (or 200 bi-nucleated cells) were analyzed via 1000x magnification with the microscope and all observable chromosomal aberrations were counted, photographed, and resulting images saved. Table 4 depicts the dicentrics found within the metaphase spreads that were analyzed from the wild boar blood samples obtained in Fukushima, Japan.

Table 4: Dicentrics in Fukushima Wild Boar Blood Samples

Wild Boar Sample Number	Date Wild Boar Blood Acquired	Metaphase Spreads Analyzed	Dicentrics Found
160610-1	160610	115	0
161208 (0-236)	161208	117	1
161208 (0-237)	161208	75	0
161209 (0-239)	161209	200	1
161209 (0-240)	161209	200	2
161209 (0-242)	161209	200	0
161213 (0-246)	161213	200	0
161215 (T-1)	161215	200	0
161215 (T-2)	161215	200	0
161216 (0-247)	161216	200	0
161216 (F-117)	161216	200	0

Figure 6 depicts a dicentric and corresponding acentric fragment from a wild boar blood sample obtained from the Fukushima Prefecture in Japan.

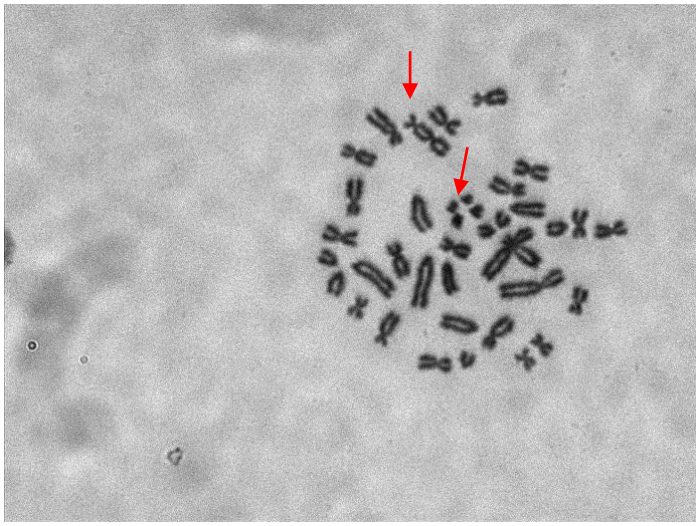

Figure 6: Dicentric in Metaphase Spread from Pig 161209 0239 from Fukushima Prefecture, Japan

Fukushima Wild Boar Micronuclei Biodosimetry

Table 5 depicts the micronuclei found within the bi-nucleated cells that were analyzed from the wild boar blood samples obtained in Fukushima, Japan.

Table 5: Micronuclei in Fukushima Wild Boar Blood Samples

Wild Boar Sample Number	Date Wild Boar Blood Acquired	Bi-nucleated Cells Analyzed	Micronuclei Found
160801-1	160801	200	0
160804-1	160806	200	0
161206 (0-228)	161206	200	1
161206 (0-229)	161206	200	0
161206 (0-231)	161206	200	2

Figure 7 depicts micronuclei emerging from a bi-nucleated cell during cell division from a wild boar blood sample obtained from the Fukushima Prefecture in Japan.

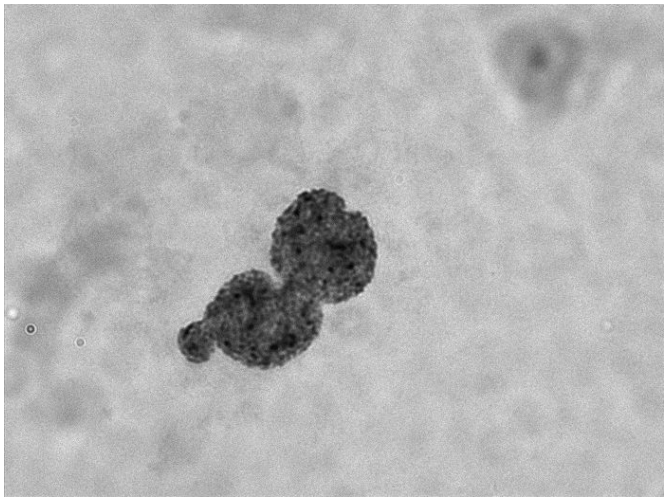

Figure 7: Micronuclei in Bi-nucleated Cell from Pig 161206 (0-231) from Fukushima Prefecture, Japan

DISCUSSION

Data Analysis

A limited number of dicentrics were observed for all the irradiated control wild boar blood samples (Table 2). The wild boar blood samples irradiated at 2 Gy also had minimal dicentrics observed in the metaphase spreads processed from the wild boar blood samples. Several wild boar blood samples were not scored for dicentrics due to inadequate staining or too few metaphase spreads. Due to the limited number of observed dicentrics in all of the samples, a dose response curve for the low radiation doses could not be established (Figure 1). The expected result was not observed as the wild boar blood samples irradiated at 2 Gy were anticipated to contain dicentric chromosomal abnormalities in every sample irradiated at 2 Gy.

Micronuclei were virtually nonexistent in all the irradiated control wild boar blood samples (Table 3). While many micronuclei samples were not scored due to the non-existence of bi-nucleated cells, the samples that were scored represented all the irradiation doses (0 Gy, 0.1 Gy, 0.2 Gy, 0.3 Gy, 0.5 Gy, 1 Gy, and 2 Gy). Since there was limited micronuclei formation, a dose response curve for the low radiation was not created (Figure 3). The expected result was again not observed for the micronuclei assessment as the wild boar blood samples irradiated at 2 Gy were anticipated to contain micronuclei chromosomal abnormalities.

Very few dicentrics were observed for all the wild boar blood samples obtained in the Fukushima Prefecture in Japan (Table 4). While a higher number of metaphase spreads were scored (200 metaphase spreads) in comparison to the control wild boar blood sample analysis (100 metaphase spreads), additional dicentrics were not observed. Also, a limited number of micronuclei was observed for all the wild boar blood samples obtained in the Fukushima Prefecture in Japan (Table 5).

Japan and USA Laboratory Work Difficulties

Due to differences in equipment and lack of adequate supplies, there were multiple laboratory procedures that were difficult to conduct in Japan, and there are several recommendations for improving future studies. As all of the wild boar blood processing protocols require the wild boar blood to be at room temperature prior to processing, the timetable for acquiring the wild boar blood and the commencement of the laboratory protocols was vital. The wild boar blood was obtained by IER researchers at 1000 hrs in the morning and would not arrive at the IER laboratory until 1800 hrs in the evening. The lag time between wild boar blood acquisition from wild boar that had been euthanized and white blood cell processing caused the wild boar blood to clot. The clotting in the blood would reduce the ability of wild boar blood cells to culture properly and not produce metaphase spreads or bi-nucleated cells.

In addition to the timetable of wild blood acquisition, the method of obtaining blood from the wild boar is also vital. When the wild boar were shot, most of the blood would exit the wound onto the ground and be unusable for laboratory analysis. The wild

boar that were electrocuted provided an adequate amount of blood for analysis of dicentric and micronuclei chromosomal abnormalities. While obtaining wild boar blood from the chest cavity also provided more blood than the carotid artery in the neck, the wild boar blood from the chest cavity produced mold within the samples during cell culturing and resulted in the incubator being infested with mold. The moldy samples were discarded as cell stimulation could not take place. The wild boar blood should only be obtained from an available artery or vein in the wild boar and not the chest cavity to reduce mold contamination in both the cell cultures and within the laboratory incubator.

Another method to reduce mold contamination is to properly mix the media mixture by adding the Fetal Bovine Serum (FBS) and the antibiotic-antimycotic to the original 500 mL RPMI media bottle, mixing the components by inverting the tightly sealed bottle, and filtering the mixture with a filter attached to a laboratory integrated vacuum. There was no laboratory vacuum system in the IER and an external vacuum pump had to be adjusted to provide suction for all the laboratory procedures. The vacuum pump was ineffective at times and should be replaced with a better system. Another system for reducing mold contamination is the laboratory hood. Ideally, a microbiology laboratory hood with positive pressure air flow should be utilized for wild boar blood processing to reduce mold contamination. The IER laboratory hood that was utilized was not providing positive pressure or negative pressure airflow which could have contributed to mold contamination in the cell cultures.

Due to the lack of blood processing equipment in the IER in Japan, all the experimental equipment and chemicals were ordered and delivered to the IER two weeks prior to the arrival of the student researchers. The laboratory equipment and chemicals must be functioning and thawed prior to the acquisition and processing of the wild boar blood. Additionally, all the laboratory chemicals to be used in the foreign laboratory must be thoroughly researched and processing protocols analyzed prior to arrival in the foreign laboratory. For example, both the mitogens PMA and Inomocyin arrived at the IER laboratory in sealed vials, without instructions on how to properly extract the powdery chemicals. The proper method of extraction was to inject 1 mL of DMSO into the vial, mix, re-inject, and remove via syringe. Unfortunately, 1 mL of deionized water was utilized and the mitogens were not properly homogenized in solution which resulted in the media mixture and subsequent cell culturing to not produce metaphase spreads.

To ensure the cell culture is properly growing, the appropriate flasks need to be purchased and utilized. Unfortunately, due to language barriers between the research students and the Japanese IER staff, flasks that had the option to be non-vented were purchased and were accidentally sealed during several blood sample processing attempts which caused the cells to die. In future studies, only vented flasks should be utilized in order to avoid the error. Another error to avoid is to determine the appropriate speed settings of the IER centrifuges. According to protocols, the metaphase spread samples and the bi-nucleated cell sample were to be centrifuged at 1000 RPM and 500 RPM, respectively. However, all of the centrifuges at the IER did not separate the cells

into pellet form from the solution unless speeds of 1700 RPM and 900 RPM were used. The speed difference may be a deviation with the Japanese centrifuges in comparison to the American centrifuges.

Another deviation in equipment was the utilization of the IER microscope and camera. The microscope at the IER allowed for adequate viewing of the chromosomal aberrations at 1000x magnification, however, the camera attached to the microscope was out of focus and would not save clear images of the dicentrics or micronuclei. Future researchers should ensure the microscope camera can effectively take and store images at 1000x magnification. Future researchers should also confer with the IER staff in utilizing the 1000x magnification on the microscope without a coverslip on the slide. While the coverslip on the slide provides a barrier between the microscope lens and the cells making cleaning much easier with an appropriate microscope lens cleaner, the coverslip and the DPX mounting serum also create an additional barrier between the chromosomal aberrations and the microscope which can interfere with focusing the microscope lens clearly to observe dicentrics or micronuclei.

The most effective method for counting dicentrics and micronuclei on a slide involves omitting the application of the coverslip and mounting media. If the processed wild boar blood samples can be scored with the IER microscope and recorded with the IER microscope camera in Japan (with the approval of the IER staff in use of the microscope without a slide coverslip and mounting media), the slides will not have to be shipped to the USA under the USDA permit guidelines to be sealed with a coverslip.

The wild boar blood obtained in Japan will be more easily scored for chromosomal aberrations without a coverslip and mounting media if the microscope analysis was conducted in Japan instead of the USA.

In addition to proposed changes to laboratory equipment and protocols for working in a foreign country, a few suggestions are provided for future studies involving wild boar irradiated blood at CSU in the USA. Some of the control wild boar blood samples (wild boar 1) were processed via the metaphase cell stimulation protocol (Appendix C) and in addition 5% glutaraldehyde was added. The purpose of the addition was to determine if following the USDA protocol for shipping frozen, fixed wild boar blood samples (prior to dropping on a slide) would be feasible from an experimental standpoint. Unfortunately, the addition of the glutaraldehyde resulted in the inability of the cellular membranes of the cells to open after the cells were dropped onto a slide and no metaphase chromosomes were visible. While seven control wild blood samples could not be counted for dicentrics as no metaphase chromosomes were visible, the knowledge that the addition of glutaraldehyde would make the metaphase cell stimulation protocol (Appendix C) ineffective was vital as all wild boar blood samples obtained in Japan were required to be dropped onto slides and sealed with a coverslip prior to shipment. The experimentation on the control wild boar blood samples determined that no frozen, fixed wild boar blood samples could be shipped from Japan as the lack of glutaraldehyde would put the shipment in violation of the USDA permit.

The control irradiated blood samples should not be processed in a large amount at one time. Instead of processing 14 variations of blood from six wild boar (84 total samples) in one day, at most, 14 samples from one wild boar should be processed in one day. During the final day of cell culture and blood processing, the specific chemicals that need to be added to each sample are time dependent and deviations by an hour or more affect the number of white lymphocytes obtained in metaphase spread and bi-nucleated cell form. When only one laboratory hood is available and one researcher is processing 84 blood samples, deviations of over two hours or more occur in the processing of the samples. The time deviations in processing the control micronuclei samples in the pilot study, caused many micronuclei samples to not properly exhibit bi-nucleated cells and resulted in the loss of data.

Implications of Results

The wild boar in the Fukushima prefecture in Japan are not consistently exposed to the same low levels of radiation due to the large roaming habitat of the wild boar which encompass variable levels of radiation and include areas that have been remediated by the Japanese government. Additionally, the wild boar may be more resilient to radiation exposure than other animals and may not readily exhibit chromosomal aberrations from low-level radiation exposure. Large variability within and across different wild boar species is possible and can affect radiation exposure biomarkers. The repair mechanisms of DNA damage may be more robust in certain wild boar species or in all wild boar overall. Some wild boar species and some wild boars

within each species can give lower discernible numbers of biomarkers of radiation exposure.

While the dose of 2 Gy should have resulted in a consistent increase in both dicentrics and micronuclei formation, the data obtained did not show a consistent increase in chromosomal aberration formation. As there is no published literature on the formation of radiation biomarkers in wild boar blood after radiation exposure, future research is necessary to determine the multiple possibilities producing the data obtained in the pilot study. The wild boar as a species may have robust DNA repair mechanisms that allow for the accurate and rapid repair of chromosomes damaged by radiation. The radiation dose of 2 Gy is not high enough to cause DNA damage in wild boar and higher radiation doses may be used to create a radiation dose response curve. The sample sizes utilized in the pilot study were too small to determine a statistically significant increase in chromosomal aberrations after radiation exposure and larger samples sizes should be employed. Wild boar blood samples should be obtained from a wide variety of wild boar species and chromosomal aberrations should be analyzed after irradiation to determine if the specific species of wild boar blood irradiated in the pilot study provided less visible biomarkers of radiation exposure than other wild boar species.

The scoring of dicentrics and micronuclei chromosomal aberrations are not the ideal method for dose assessment in wild boar due to lack of observable chromosomal aberrations. In the absence of more robust dose-response data it will not be possible to

reduce the uncertainties of assessing the environmental impacts from releases of nuclear facilities, nor will it be possible to confidently create appropriate safeguards that protect humans and the environment from low-dose, chronic exposures to radiation (Hinton et al. 2013). If wild boar do not portray biomarkers of radiation, biodosimetry is not an ideal method for radiation dose assessment to the wild boar.

Future Studies

Unfortunately, there is a lack of published literature on wild boar chromosomal dicentric and micronuclei aberration investigations and there have been no current published studies involving the irradiation of wild boar blood for biodosimetry analysis. The dicentric analysis technique with the creation of a dose-response curve to estimate radiation dose is considered the gold standard for biodosimetry (Hall et al. 2011). For future studies, the feasibly of utilizing dicentrics and micronuclei as biomarkers of radiation exposure in wild boar should be investigated with the use of higher doses of radiation (higher than 2 Gy). DNA translocations should also be researched to determine if other biomarkers more sensitive than dicentrics or micronuclei can be utilized to better estimate radiation dose to wild boar. Additionally, a higher number of wild boar blood samples should be obtained and a larger number of metaphase spreads and bi-nucleated cells should be analyzed in order to determine if the results obtained are consistent with the results observed in the pilot study.

The biological effects from a one-time, discrete radiation exposure may not reflect the same biological effects from a long-term exposure due to radiation hormesis

or other overactive biological repair mechanism from constant radiation exposure. A dose-response curve created from short radiation exposures may not represent a dose obtained from long-term exposure to radiation. Additional research must be conducted to determine if the wild boar residing in the low-radiation level areas in the Fukushima prefecture have become more resilient to radiation exposure via comparison with long-term, irradiated blood from the same Japanese wild boar species residing in other islands in Japan.

In future studies, the radiation dose to the wild boar can be estimated using soil samples from soil collected in the habitat of the wild boar. While the current pilot study indicates less than 1% chromosomal aberrations for single radiation exposures from 0.1 Gy to 2 Gy and Fukushima wild boar, a more robust study that analyzes a higher number of metaphase cell spreads and bi-nucleated cells should be conducted to determine if results with larger sample sizes are also consistent with the pilot study results. Additionally, a long-term research study upon the variability of wild boar DNA within multiple wild boar populations needs to be conducted to quantify biomarkers in wildlife exposed to low doses of environmental radiation. The karyotyping of the Japanese wild boar is a current ongoing study that will provide much needed scientific data regarding the variability of the genes within the Japanese wild boar population in the Fukushima Prefecture and can be further utilized to determine the sample sizes needed in future studies that are adequate for the amount of variability within the Japanese wild boar species. All future studies will greatly impact the current knowledge of low level radiation exposure as consistent results with the pilot study on the lack of

radiation biomarkers from low level radiation in sentinel animal species can indicate the similarity with homo sapiens during human habitation in an environment with low level, lingering radiation.

CONCLUSION

The purpose of the quantifying biomarkers in wildlife exposed to low doses of environmental radiation pilot study is to evaluate if wild boar blood biomarkers could be utilized to determine radiation dose. Two techniques for analyzing biomarkers of effects were utilized to determine the extent of the effects of the constant, low-dose radiation exposure within the Japanese wild boar. Due to the limited number of observed chromosomal aberrations in both the Fukushima wild boar blood samples and the irradiated control wild boar blood samples from Kentucky USA, a dose-response relationship was not validated between radiation dose and dicentric or micronuclei biomarkers of radiation.

Obtaining wild boar blood samples from a related species of wild boar occupying Kentucky and irradiating the obtained blood samples did not result in the creation of a dose-response curve for biomarkers of low level exposure to radiation. While the procedures for initiating and analyzing biomarkers of radiation exposure in wild boar DNA are effective, the hypothesis of the pilot study was not proven valid. Chronic radiation dose to wild boar exposed to low levels of lingering environmental ionizing radiation in the Fukushima prefecture as well as in irradiated blood from wild boar residing in areas experiencing only natural background radiation will require further investigation. The low dose radiation exposures do not appear to affect the wild boar DNA with discernible chromosomal aberrations. Future studies need to be conducted to ascertain the dose to the wild boar required to increase the number of dicentrics and

micronuclei. Future studies that yield similar results to the pilot study can indicate the ability of humans to habitat areas of low level, lingering radiation with no resulting biomarkers of radiation exposure.

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APPENDIX A: IACUC APPROVAL

Research Integrity & Compliance Review Office
Office of Vice President for Research
208 University Services Center
2011 Campus Delivery
Fort Collins, Colorado 80523- 2011
TEL: (970) 491- 1553
FAX: (970) 491- 2293
http://ricro.research.colostate.edu

To: Nadia Halim, Thomas Johnson, Susan Bailey

From: Research Integrity and Compliance Review Office (RICRO)

Date: May 5, 2016

RE: IACUC Exemption of "Quantifying Biomarkers in Wildlife Exposed to Low Doses of Environmental Radiation"

This is to inform you that your IACUC Exemption request for "Quantifying Biomarkers in Wildlife Exposed to Low Doses of Environmental Radiation" has been reviewed by RICRO and the Attending Veterinarian (or his delegate), and is exempt from IACUC oversight. Therefore, an IACUC protocol does not need to be submitted for these activities.

If there are any changes in this project, please submit changes via the IACUC Exemption Form to ensure that this exemption is still valid prior to implementation

Thank you for your diligence in the care and use of animals at CSU. Good luck with your project.

Sincerely,
Research Integrity and Compliance Review Office (RICRO)

Cc: Terry Engle, PhD, IACUC Chair
Lon Kendall, DVM, PhD, CSU Attending Veterinarian
Karen Dobos, PhD, RICRO Director

APPENDIX B: MEDIA PREPARATION FOR METAPHASE AND BI-NUCLEATED CELL PREPARATION

1. Using 10 mL syringe with a 27 gauge needle, inject 1 mL of DMSO in PMA vial. The PMA vial has 1 mg of PMA in powder form.

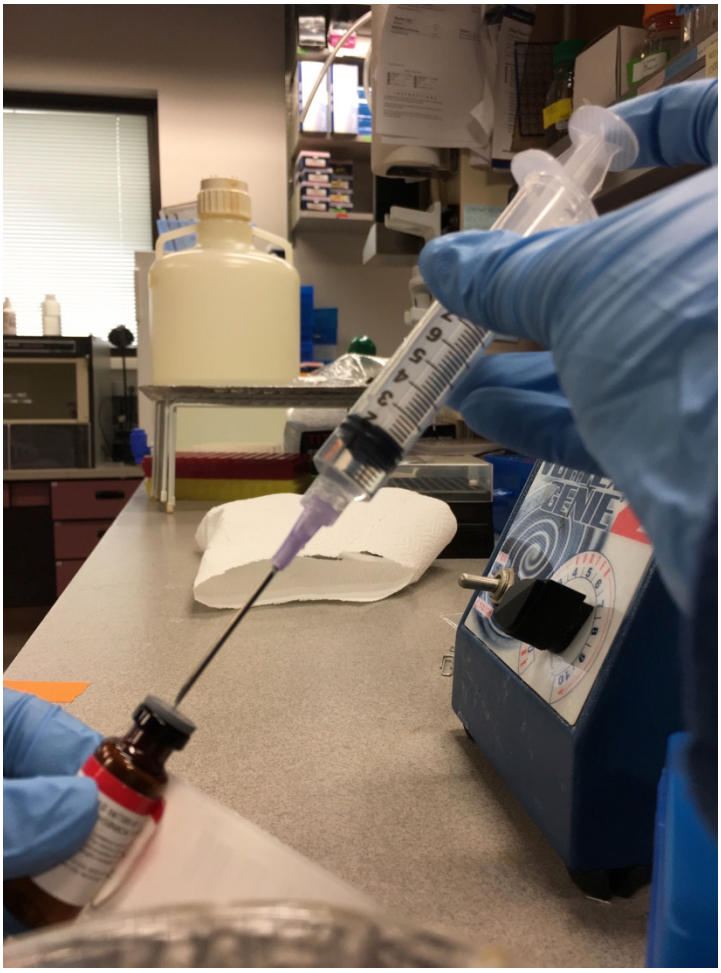

Figure 8: Injecting 1 mL of DMSO into PMA vial

   a. Invert vial with DMSO and PMA powder to ensure mixture is homogeneous

   b. Re-inject syringe into vial and remove PMA mixture

c. Separate all PMA into 4 µL allocates each, place into tiny conicals, wrap in aluminum foil to avoid UV light, and place into -20°C (freezer)

2. Using 10 mL syringe with a 27 gauge needle, inject 1 mL of DMSO in Ionomoycin vial (with 1 mg of Ionomoycin)

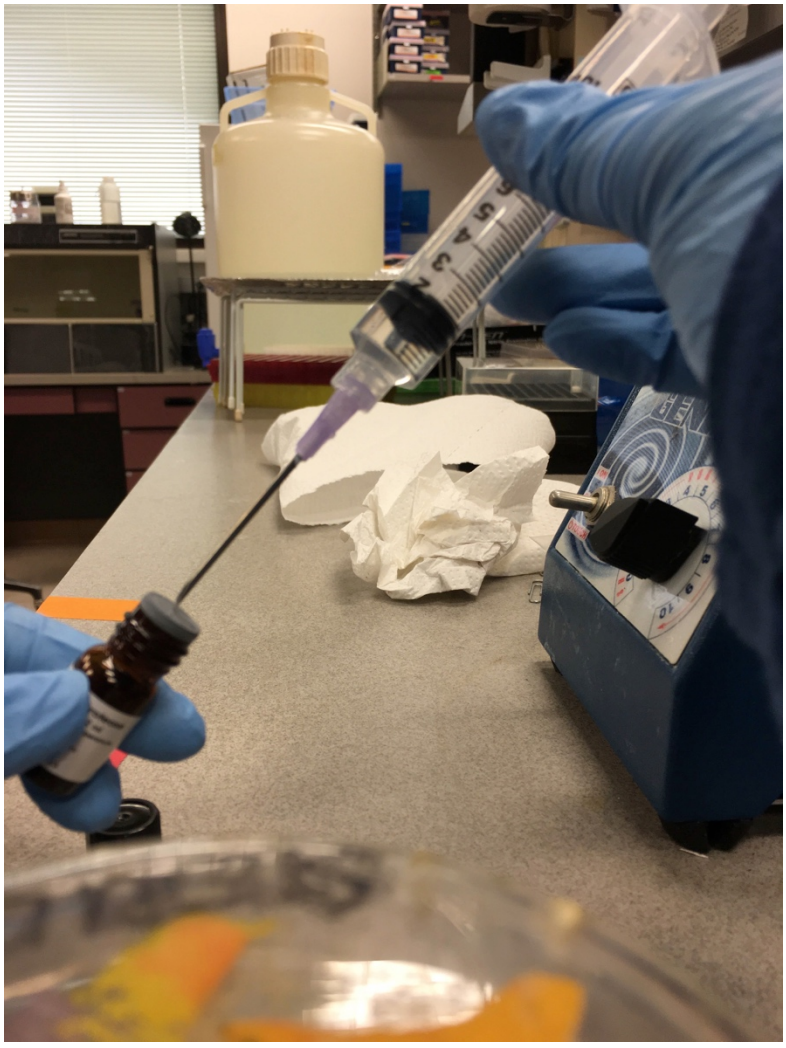

Figure 9: Injecting 1 mL of DMSO into Ionomoycin vial

a. Invert vial with DMSO and Ionomycin powder to ensure mixture is homogeneous

b. Re-inject syringe into vial and remove Ionomycin mixture

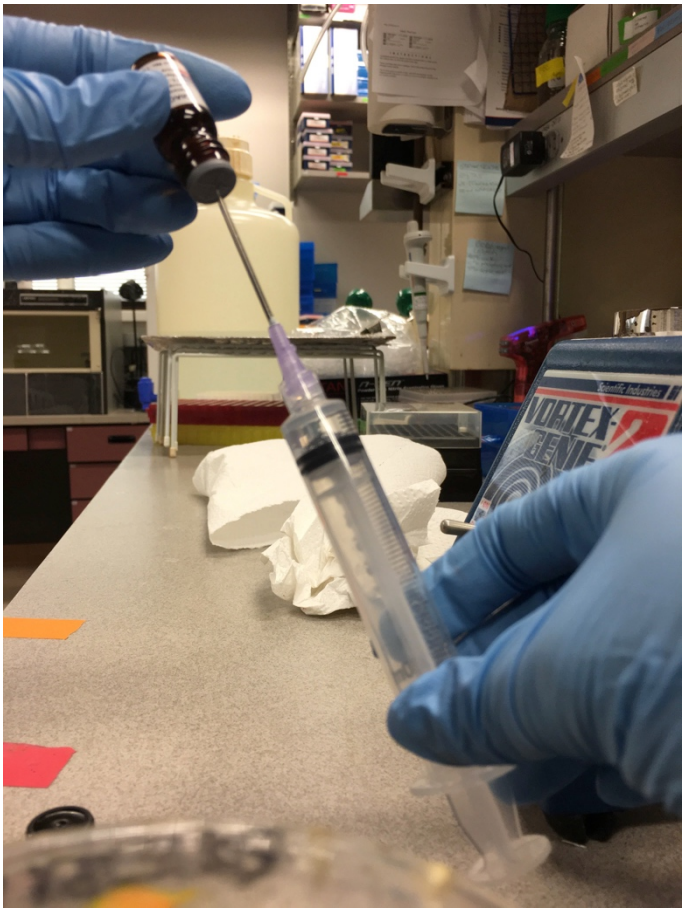

Figure 10: Re-injecting needle into Ionomoycin vial and removing mixture

b. Separate all Ionomycin into 40 µL allocates each, place into tiny conicals, wrap in aluminum foil to avoid UV light, and place into -20°C (freezer)

3. RPMI-1640 medium supplemented with 1% L-glutamine should be purchased from Sigma Scientific.

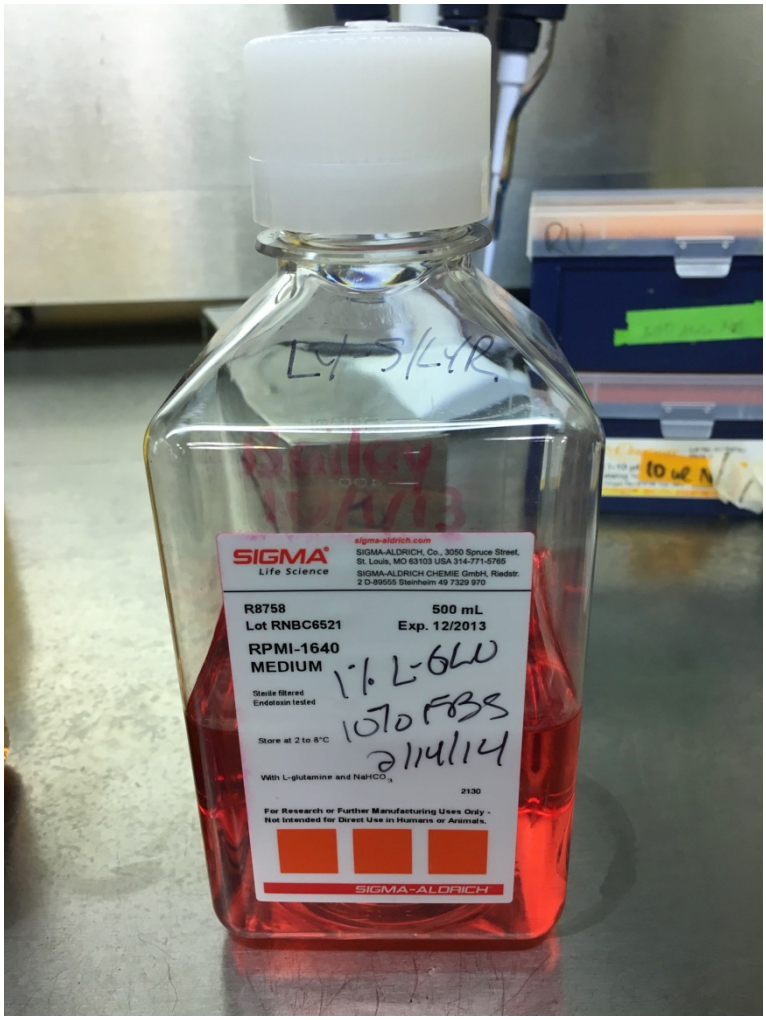

Figure 11: Image of RPMI-1640 medium supplemented with 1% L-glutamine

4. Create prepared medium

    a. Thaw FBS which has been previously frozen at -20°C.

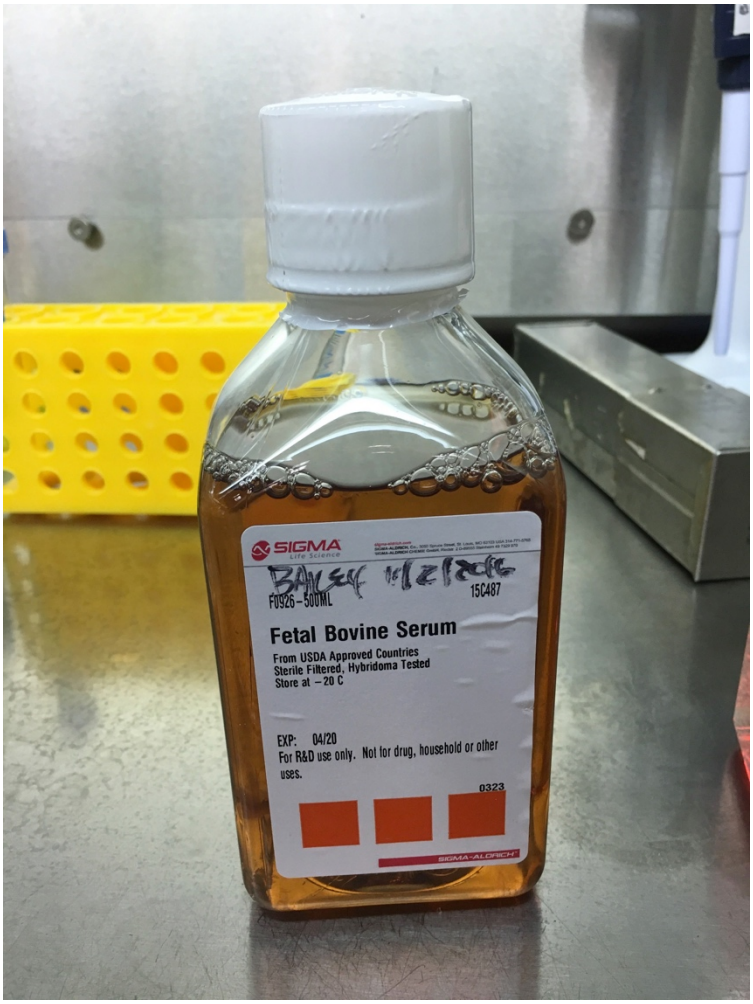

Figure 12: Image of FBS

b. Add 217 mL of FBS to 500 mL of RPMI-1640 medium already bought supplemented with 1% L-glutamine in original media bottle

c. Add 7 mL of antibiotic-antimycotic

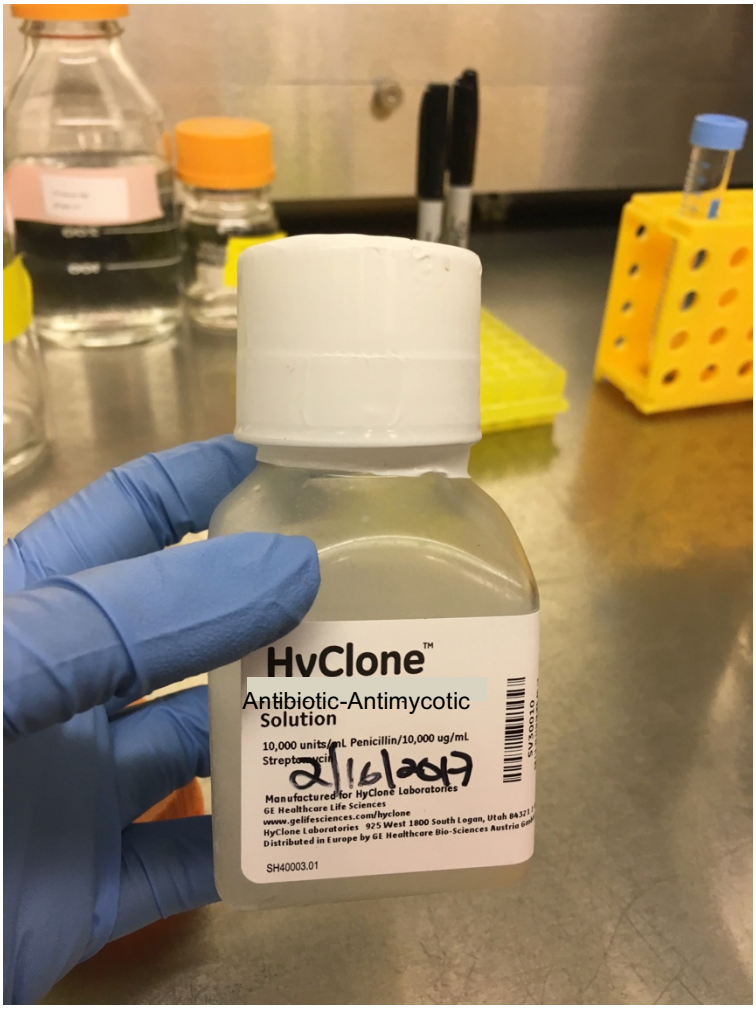

Figure 13: Image of antibiotic-antimycotic

d. Ensure final concentrations of media is RPMI-1640 medium supplemented with 1% L-glutamine, 30% FBS and 1% antibiotic-antimycotic

e. Swirl media mixture in bottle

f. Filter media using media filter (attached to a glass bottle) with supplied air hose connected to a vacuum

Figure 14: Image of media filtration setup

5. Allocate 35 mL of mixed media in 50 mL conical tubes each and freeze in -20°C.

APPENDIX C: WILD BOAR BLOOD STIMULATION FOR METAPHASE CHROMOSOME PREPARATION

1. Thaw two 50 mL conical tube aliquot of medium

2. In a T-25 vented flask, add 1mL whole blood from heparinized blood collection vial to 9mL prepared medium.

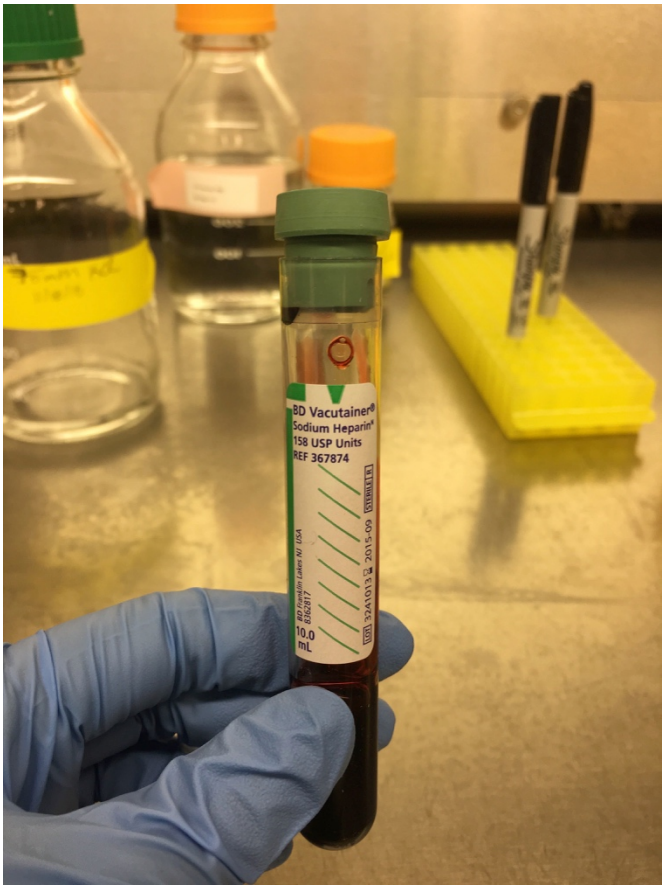

Figure 15: Wild boar blood in heparinized blood vial

Figure 16: T-25 vented flask with whole blood and prepared medium

3. Add 0.5 µL of 1 mg/mL PMA and 11 µL of 1 mg/mL of Ionomycin to T-25 flask ensuring (ensure final concentration of PMA and Inonomycin in each T-25 vented flask are as follows 50 ng/mL PMA, 1 µg/mL Ionomycin).

4. For PMA, stock PMA concentration is 1 mg/mL = 1 µg/µL

    a. Ensure PMA added is at 50 µg/mL

    b. Total cell culture volume is 11 mL

    c. Need 0.5 µg of PMA from stock solution

i. (50 ng/mL)*(11 mL) = 550 ng = 0.5 µg

d. (0.5 µg)/(1µg/uL) = 0.5 µL of PMA is needed per sample

5. For Ionomycin, stock Ionomycin concentration is 1 mg/mL = 1 ug/uL

    a. Ensure Ionomycin added is at 1 µg/mL
    b. Total cell culture volume is 11 mL
    c. Need 11 µg of Ionomycin from stock solution

        i. (1 µg/mL)*(11 mL) = 11 µg

    d. (11 µg)/(1 µg/uL) = 11 µL of Ionomycin is needed per sample

6. Incubate at 37°C and 5% CO_2 for 68 hours in incubator (name and type)

7. Add 110 µL colcemid (purchased from Sigma SKU) and incubate an additional 4 hours.

    a. Stock colcemid concentration is 10 µg/mL
    b. Ensure colcemid added is at 0.1 µg/mL or 10 µl/mL
    c. Total cell culture volume is 11 mL
    d. Need 1.1 µg of colcemid from stock solution

        i. (0.1 µg/mL)*(11 mL) = 1.1 µg

    e. (1.1 µg)/(10 ug/mL) = 0.110 mL = 110 µL of colcemid is needed per sample

8. After 72 hours of total incubation time, pipette the blood and medium several times to reduce clumping.

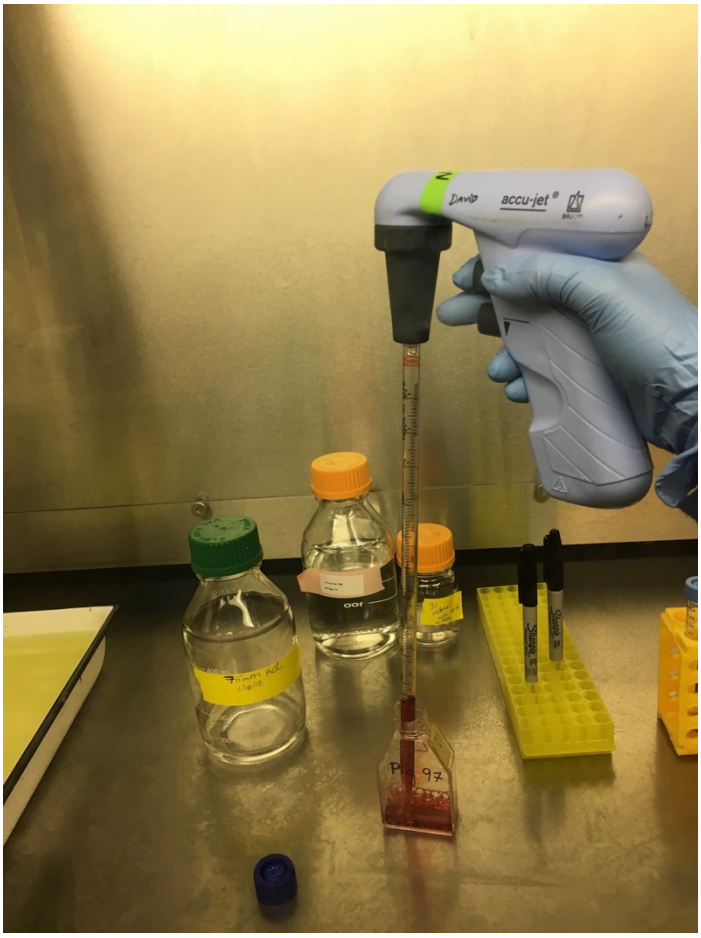

Figure 17: Pipetting of blood and medium in T-25

9. Transfer contents of each flask to a 15mL polystyrene conical and centrifuge at 1000 rpm for 5 minutes.

10. Aspirate off medium and suspend pellets by gently flicking the conical.

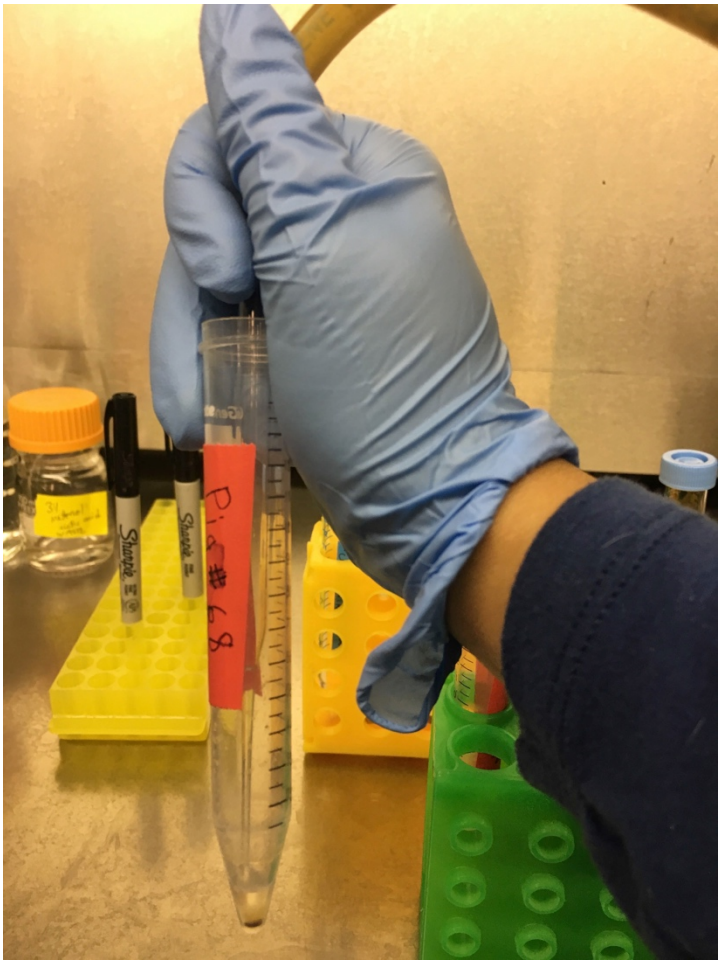

Figure 18: Aspirating off the medium to see pellet

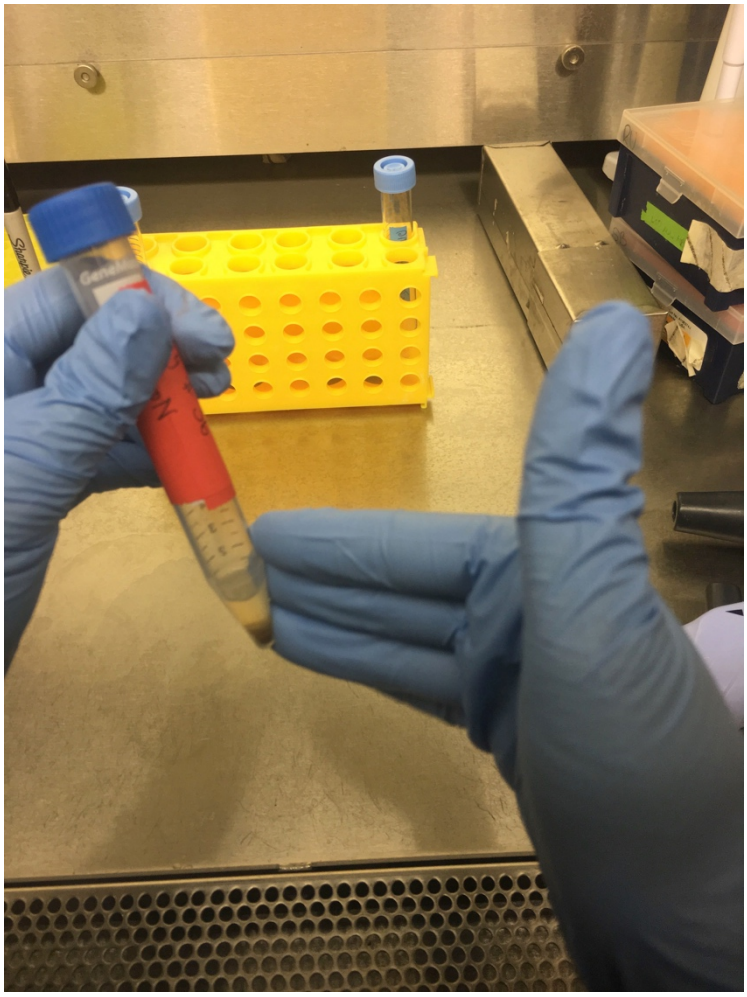

Figure 19: Flicking conical to suspend pellet

11. Add 6mL of 75 mM KCl (5.59 g/L of dH2O) hypotonic solution and incubate 30 minutes at room temperature, inverting every 5 minutes.

12. Add 1.5 mL freshly prepared 3:1 methanol to acetic acid fixative to each conical and invert several times. Solution should turn a dark red/brown color.

13. Centrifuge at 1000 rpm for 5 minutes.

14. Aspirate supernatant off, leaving about 1.5 mL solution covering the pellet.

a. It is difficult to see pellet, so be sure to not aspirate too close to the pellet and leave at least 1.5 mL of solution covering the pellet.

15. Suspend the pellet by gently flicking the conical.

16. While vortexing (using Fisher Guide Genie Vortex 2) at low speeds, add 5 mL 3:1 methanol to acetic acid fixative. Add the first 2mL drop wise to minimize clumping.

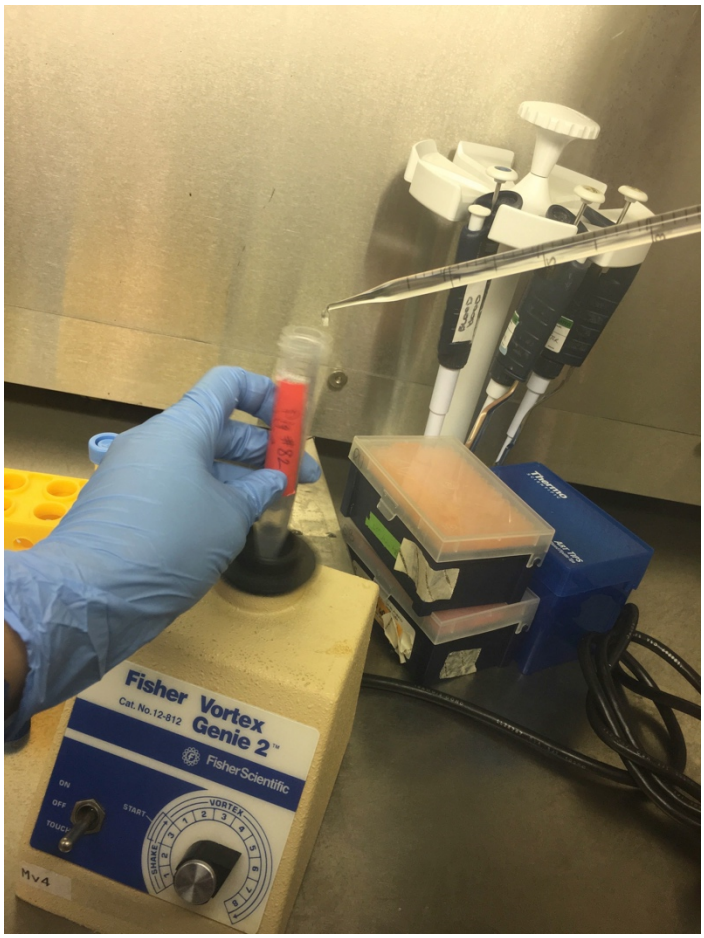

Figure 20: Adding 5 mL 3:1 methanol to acetic acid fixative while vortexing

17. Pre-fix at room temperature for 20 minutes. At this point the conicals may be stored at -20°C until ready to fix the pellets.

18. Centrifuge conicals at 1000 rpm for 10 minutes.
19. Aspirate off all fixative, leaving about 0.5 mL covering the pellets.
20. Suspend the pellets by gently flicking the side of the conicals.
21. Add 5 mL fixative while vortexing at low speeds then centrifuge at 1000 rpm for 10 minutes.
22. Aspirate off all fixative, leaving about 0.5 mL covering the pellets.
23. Suspend the pellets by gently flicking the side of the conicals.
24. Add 5 mL fixative while vortexing at low speeds then centrifuge at 1000 rpm for 10 minutes.
25. Aspirate off all fixative, leaving about 0.5 mL covering the pellets.
26. Suspend the pellets by gently flicking the side of the conicals.
27. Add 5 mL fixative while vortexing at low speeds then centrifuge at 1000 rpm for 10 minutes.
28. Repeat steps 25 - 27 until pellets do not have any red blood cells present.
29. At this point you may place the conicals at -20°C until ready to drop the cells onto slides.

APPENDIX D: WILD BOAR BLOOD METAPHASE SLIDE PREPARATION

1. Carefully place cell preparation slide with frosted side up into container with deionized water.

Figure 21: Cell Slide inside deionized water container

2. Place water container with slides at -20°C for 6 minutes (inside freezer).

3. Remove slide from cold, deionized water and carefully label with a pencil

4. Place slide angled up on paper towel

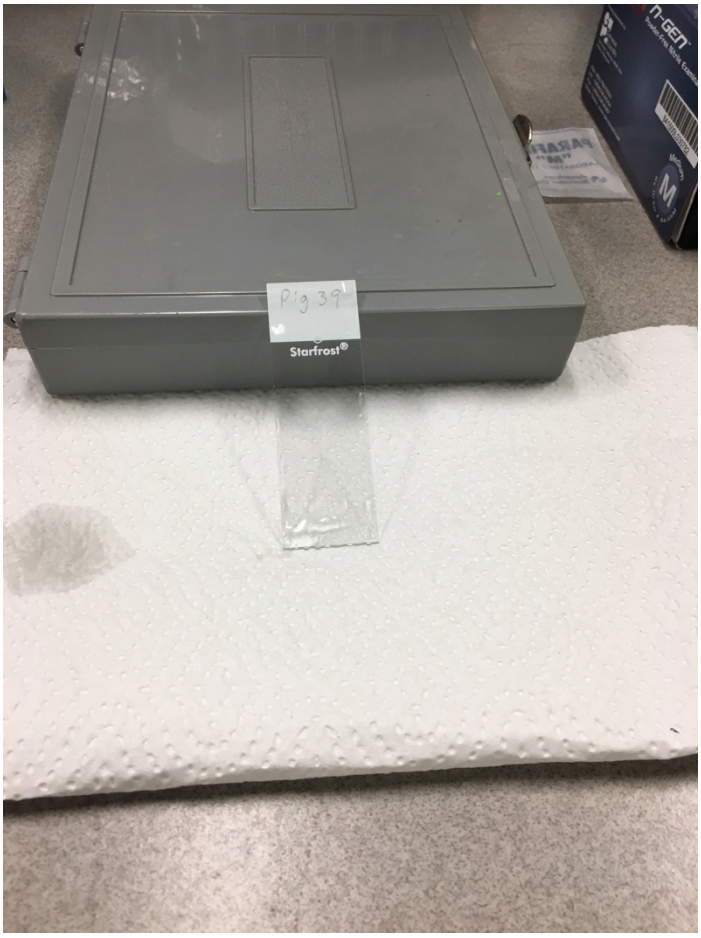

Figure 22: Cold deionized water slide angled up on paper towel

5. Flick conical to re-suspended pellet

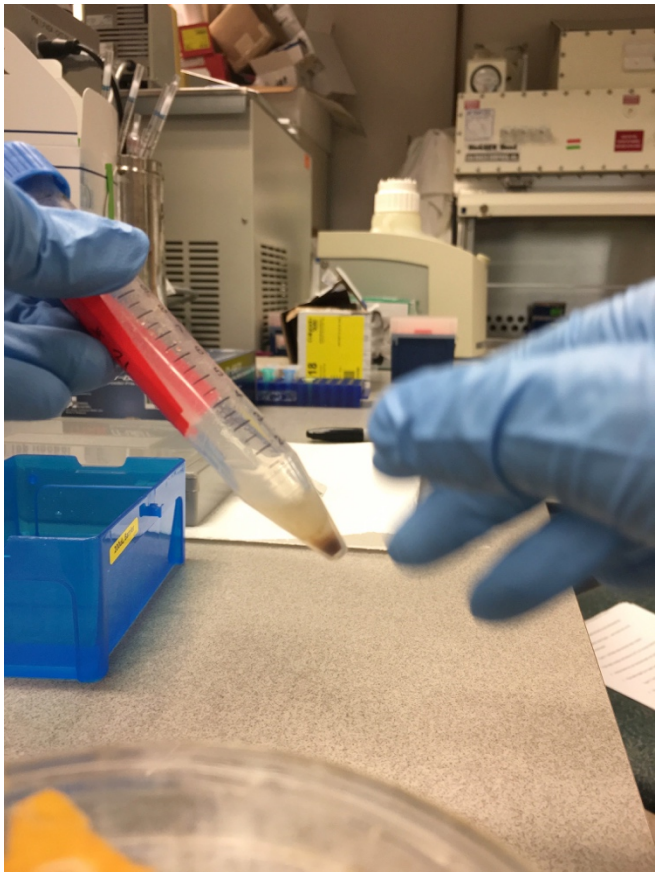

Figure 23: Flicking conical to re-suspend pellet

6. Remove 0.5 mL of re-suspended pellet with 3:1 methanol:acetic acid fixative via Pasteur pipette and Pasteur pipette bulb.

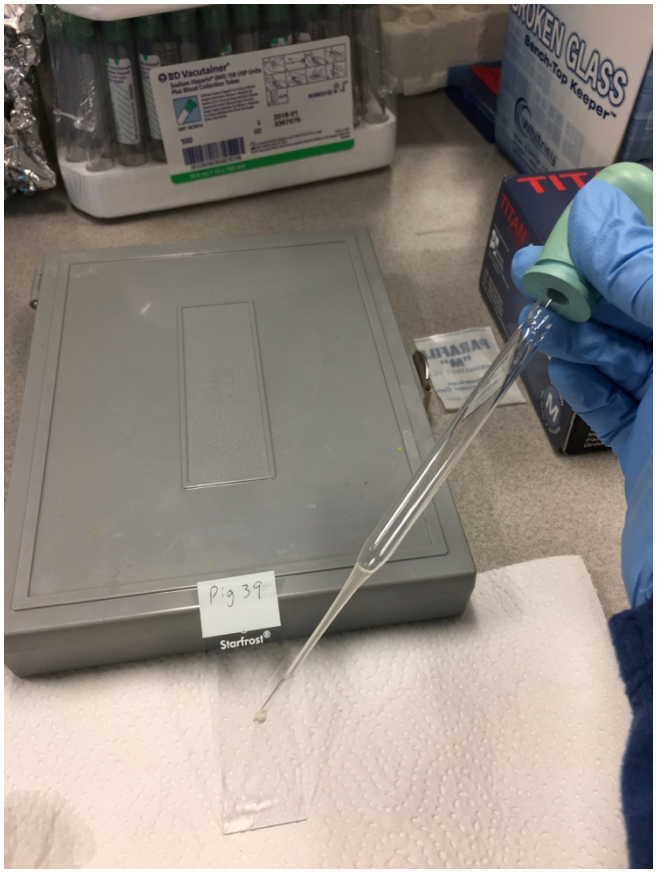

Figure 24: Cells inside pipette ready to be dropped

7. Drop cells slowly across slide horizontally starting from area underneath frosted portion.

    a. Drop 3 drops horizontally on top of slide, 3 drops in middle of slide horizontally, and drop 2 drops near bottom of slide

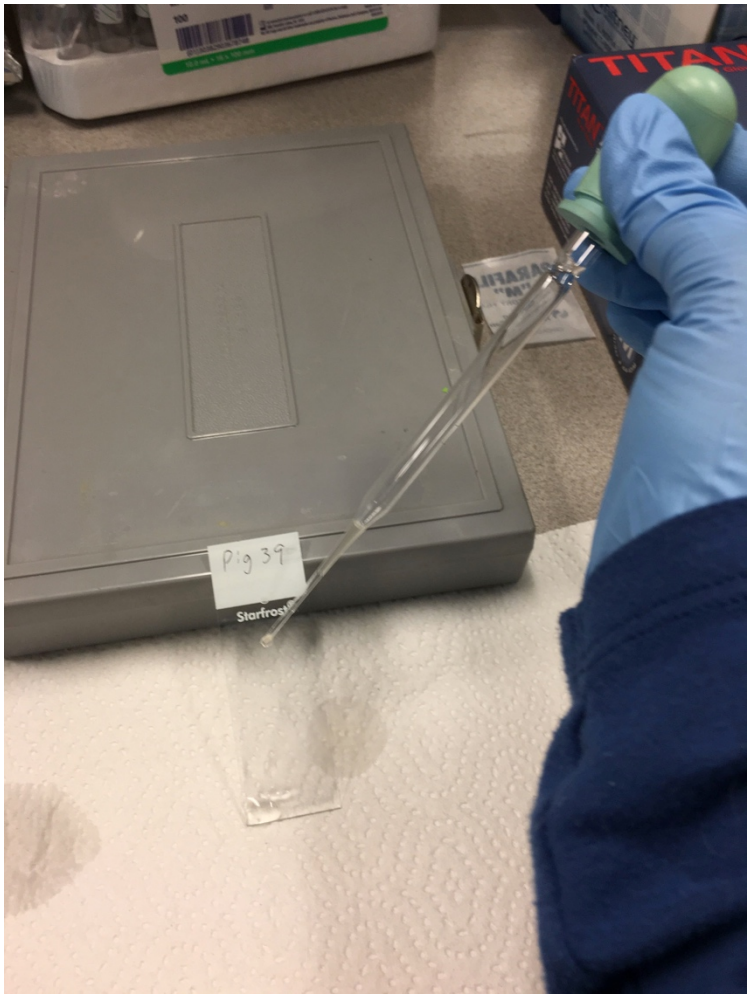

Figure 25: Drop cells onto slide

8. Let slides air dry completely until no moisture is visible.

9. Store slides for at least 24 hours inside humidity chamber

Figure 26: Slides inside humidity chamber

a. Cover humidity chamber

10. After 24 hours, remove slides from humidity chamber and commence Appendix G

APPENDIX E: BI-NUCLEATED CELL CULTURE PREPARATION FROM WILD BOAR WHOLE BLOOD

1. Thaw two 50 mL conical tube aliquot of medium
2. In a T-25 vented flask, add 1mL whole blood from heparinized vial to 9mL prepared medium.
3. Add 0.5 µL of 1 mg/mL PMA and 11 µL of 1 mg/mL of Ionomycin to T-25 flask ensuring (ensure final concentration of PMA and Inonomycin in each T-25 vented flask are as follows 50 ng/mL PMA, 1 µg/mL Ionomycin).
4. For PMA, stock PMA concentration is 1 mg/mL = 1 µg/µL
    a. Ensure PMA added is at 50 µg/mL
    b. Total cell culture volume is 11 mL
    c. Need 0.5 µg of PMA from stock solution
        i. (50 ng/mL)*(11 mL) = 550 ng = 0.5 µg
    d. (0.5 µg)/(1µg/uL) = 0.5 µL of PMA is needed per sample
5. For Ionomycin, stock Ionomycin concentration is 1 mg/mL = 1 ug/uL
    a. Ensure Ionomycin added is at 1 µg/mL
    b. Total cell culture volume is 11 mL
    c. Need 11 µg of Ionomycin from stock solution

i. (1 µg/mL)*(11 mL) = 11 µg

d. (11 µg)/(1 µg/uL) = 11 µL of Ionomycin is needed per sample

6. Incubate at 37°C and 5% CO_2 for 44 hours in incubator (name and type)

7. At 44 hours, add 6.6 µL of Cytochalasin B from 5 mg/mL stock concentration of Cytochalasin B

    a. Stock Cytochalasin B was created by injecting 1 mL of DMSO into Cytochalasin B 5 mg vial. Stock concentration is 5 mg/mL = 5 µg/µL

    a. Ensure Cytochalasin B added is at 3 µg/mL

    b. Total cell culture volume is 11 mL

    c. Need 33 µg of Cytochalasin B from stock solution

        i. (3 µg/mL)*(11 mL) = 33 µg

    d. (33 ug)/(5 ug/uL) = 6.6 uL of Cytochalasin B is needed per sample

8. At 72 hours, transfer blood/medium to 15 ml polystyrene conical.

9. Centrifuge at 1200 rpm (300g) for 5 minutes.

10. Aspirate off medium right above red cell pellet and suspend pellet by flicking the conical.

    a. Be sure there are no cells still stuck to the bottom of the conical before adding fixative.

11. Add 5 mL freshly prepared 3:1 methanol to acetic acid.

a. At this point, slides may be stored at -5°C until ready for further processing.

12. Centrifuge at 500 rpm (~70g) for 10 minutes.
13. Aspirate leaving ~0.5 mL covering the pellet.
14. Gently flick pellet to suspend.
15. Add 5 mL freshly prepared 3:1 methanol to acetic acid.
16. Centrifuge at 500 rpm (~70g) for 10 minutes.
17. Aspirate leaving ~0.5 mL covering the pellet.
18. Gently flick pellet to suspend.
19. Add 5 mL freshly prepared 3:1 methanol to acetic acid.
20. Centrifuge at 500 rpm (~70g) for 10 minutes.
21. Aspirate leaving ~0.5 mL covering the pellet.
22. Gently flick pellet to suspend.
23. Repeat steps 18-21 until red blood cells are not present.
24. Slides may be stored at -5°C until ready for staining (Appendix G).

APPENDIX F: WILD BOAR BLOOD BI-NUCLEATED CELLS SLIDE PREPARATION

1. Carefully place cell preparation slide with frosted side up into container with deionized water.
    a. No need to use cold slides to drop cells
2. Remove slide from deionized water and carefully label with a pencil
3. Place slide angled up on paper towel
4. Gently flick conical to re-suspended pellet
5. Remove 0.5 mL of re-suspended pellet with 3:1 methanol:acetic acid fixative via Pasteur pipette with Pasteur pipette bulb.
6. Drop cells gently and slowly across slide horizontally starting from area underneath frosted portion.
    a. Drop 3 drops horizontally on top of slide, 3 drops in middle of slide horizontally, and drop 2 drops near bottom of slide
7. Let slides air dry completely until no moisture is visible.
8. Store slides for at least 24 hours at 4°C until ready to stain (Appendix G)

APPENDIX G: GIEMSA STAINING FOR CHROMOSOME ABERRATIONS

1. Dissolve 1 Gurr tablet (Sigma…) in 100 mL of deionized water in 150 mL beaker
2. Removed 47.5 mL of Gurr and deionized water solution and place into new 100 mL beaker
3. Add 2.5 mL Giemsa solution to the 47.5 mL of Gurr and deionized water solution
4. Transfer 15 mL of Giemsa solution with Gurr and deionized water into Coplin Jar
5. Fill 15 mL of deionized water into 2 clean empty Coplin jars each
6. Submerge slides (5 at a time if needed) in Coplin Jar Giemsa solution for 8 minutes

Figure 27: Coplin Jar with Giemsa staining solution with 5 slides

7. Rinse slides in fresh deionized water for 6 minutes in first, deionized-water only Coplin Jar

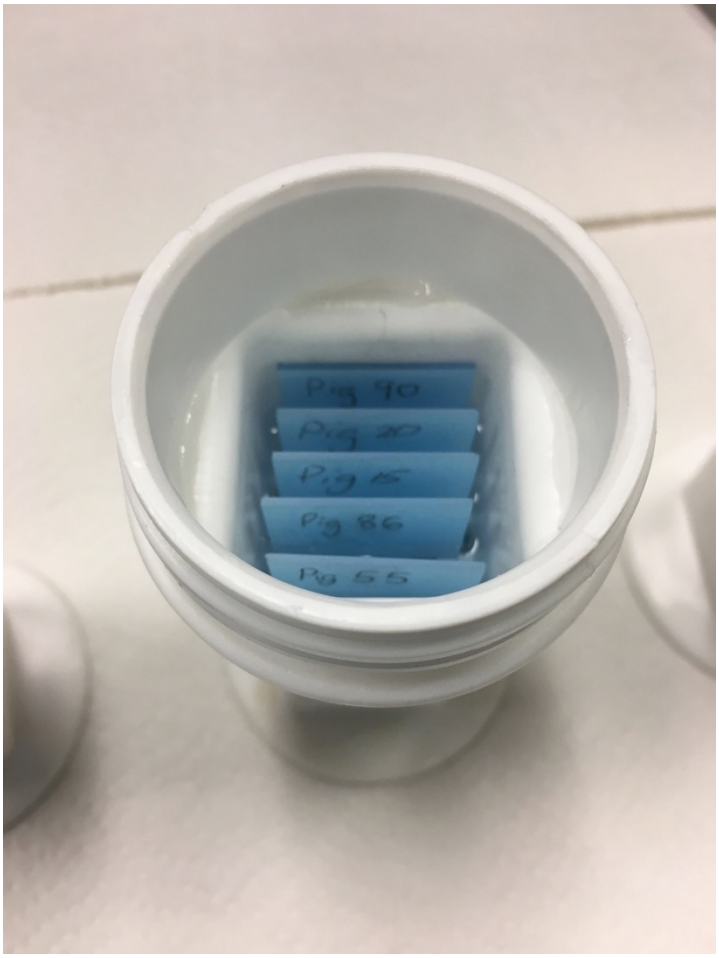

Figure 28: Coplin Jar with deionized water with 5 slides

8. Rinse slides in fresh deionized water for 4 minutes in second, deionized-water only Coplin Jar

9. Place slides onto paper towels (face-up) until dry

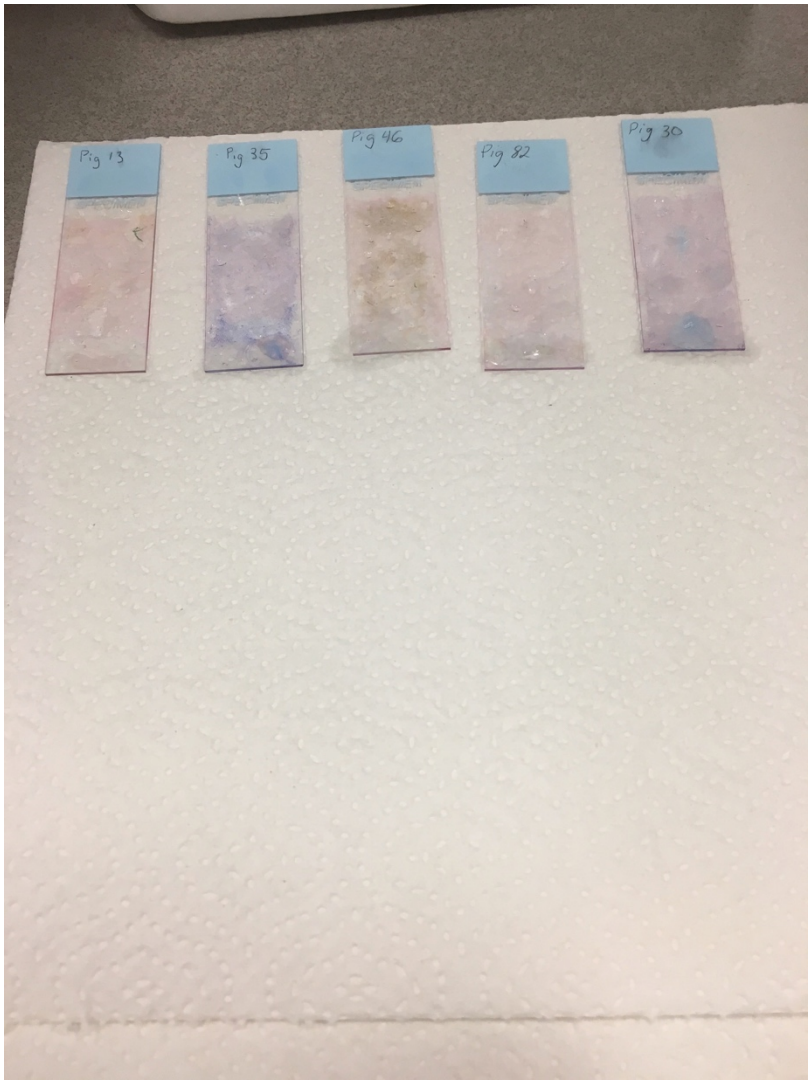

Figure 29: Drying slides

10. Place one drop of DPX Mounting Media via pipette and pasture pipette bulb onto center of front side of slide

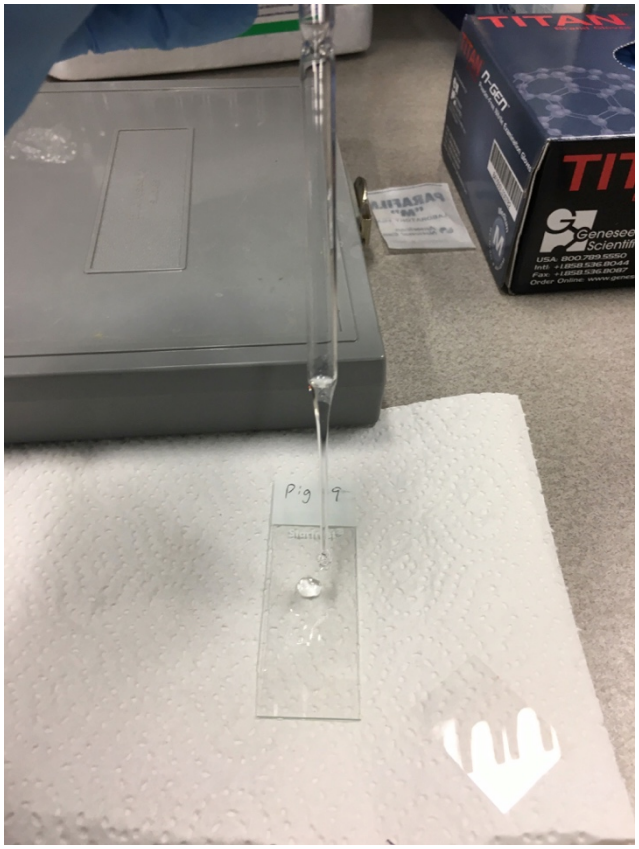

Figure 30: DPX Mounting Media drop

11. Place coverslip onto slide

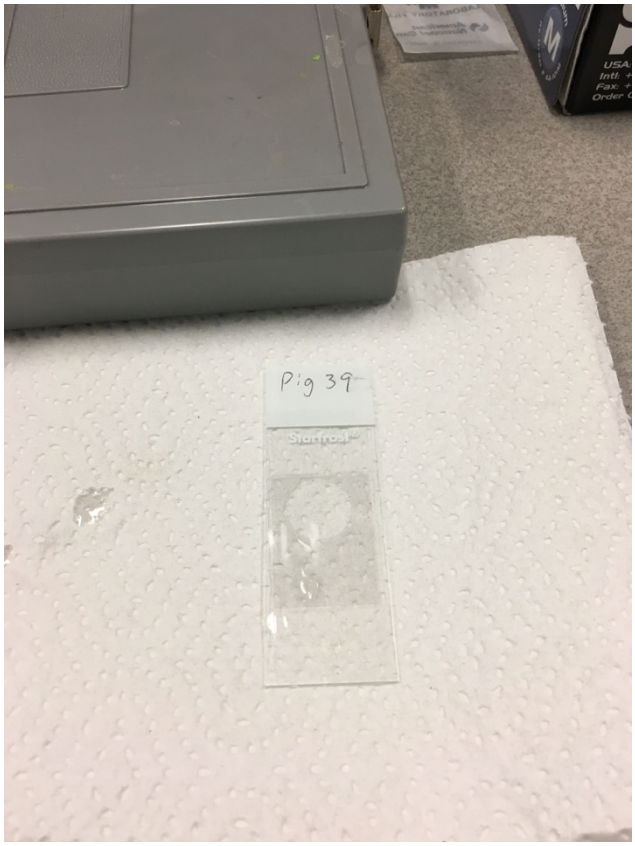

Figure 31: Coverslip on slide

12. Analyze chromosomes (or bi-nucleated cells) under a bright field microscope at 1000x total magnification for chromosomal dicentrics (or micronuclei)

13. Analyze 100 metaphase spreads (or bi-nucleated cells) per sample/dose for control samples

14. Analyze 200 metaphase spreads (or bi-nucleated cells) per sample/dose for Fukushima Wild Boar samples

APPENDIX H: USDA PERMIT

USDA

United States Department of Agriculture

Animal and Plant Health Inspection Service

Veterinary Services

National Center for Import and Export

Animal Products

4700 River Road
Unit 40
Riverdale, MD 20737

Telephone:
(301) 851-3300

FAX:
(301) 734-8226

Prof. Thomas E. Johnson / Colorado State University
Environmental and Radiological Health Science Building
1618 Campus Delivery
Fort Collins, CO 80523

Monday, December 12, 2016

Dear Prof. Thomas E. Johnson:

Your USDA Veterinary Permit to import and/or transport controlled materials, organisms, or vectors accompanies this cover letter.

Review this permit carefully, as the statements and language may have changed to reflect the requirements of newly published regulations.

Please note the following:

- USDA Veterinary Permits no longer require a signature. Use of the permit for importation of the described commodity(ies) is acknowledgement that the permittee is legally responsible for complying with the permit conditions.
- Review the import permit for errors. Should you identify any errors, please contact our office immediately
- A copy of the permit must accompany every shipment.

Do Not send the permit back to this office.

Contact our office with any questions or concerns at 301-851-3300, option 1.

Sincerely,

DShangford

Dr. Deborah Langford
Staff Veterinarian
Import Animal Products
National Import Export Services (NIES)

***USER FEES:** **New permit application $150.00, Renewal permit $97.00, Amended permit $75.00, FBS inspection $512.00 (all fees are per application) and the Import Compliance fee $565.00 per shipment.**

U.S.DEPARTMENT OF AGRICULTURE
ANIMAL AND PLANT HEALTH INSPECTION SERVICE
VETERINARY SERVICES
RIVERDALE, MARYLAND 20737
file:///D:/Inetpub/wwwroot/Epermits/images/

UNITED STATES VETERINARY PERMIT FOR IMPORTATION AND TRANSPORTATION OF CONTROLLED MATERIALS AND ORGANISMS AND VECTORS

PERMIT NUMBER
132546
Research

DATE ISSUED
12/12/2016

DATE EXPIRES
12/12/2017

NAME AND ADDRESS OF SHIPPER(S)

Various shippers within...
Japan

CC:
Service Center, CO (Lakewood, CA)

NAME AND ADDRESS OF PERMITTEE INCLUDING ZIP CODE AND TELEPHONE NUMBER

Prof. Thomas E. Johnson
Colorado State University
Environmental and Radiological Health Science Building
1618 Campus Delivery
Fort Collins, Colorado 80523
970-491-0563

U.S. PORT(S) OF ARRIVAL
DENVER, CO

MODE OF TRANSPORTATION AIR

AS REQUESTED IN YOUR APPLICATION, YOU ARE AUTHORIZED TO IMPORT OR TRANSPORT THE FOLLOWING MATERIALS

Teeth, hair, and/or blood samples (fixed and/or on slides) from wild boar (porcine origin)

RESTRICTIONS AND PRECAUTIONS FOR TRANSPORTING AND HANDLING MATERIALS AND ALL DERIVATIVES

THIS PERMIT IS ISSUED UNDER AUTHORITY CONTAINED IN 9 CFR CHAPTER 1, PARTS 94,95 AND 122. THE AUTHORIZED MATERIALS OR THEIR DERIVATIVES SHALL BE USED ONLY IN ACCORDANCE WITH THE RESTRICTIONS AND PRECAUTIONS SPECIFIED BELOW (ALTERATIONS OF RESTRICTIONS CAN BE MADE ONLY WHEN AUTHORIZED BY USDA, APHIS, VS).

- **Adequate safety precautions shall be maintained during shipment and handling to prevent dissemination of disease.**

- With the use of this permit I, Prof. Thomas E. Johnson, Permittee, acknowledge that the regulated material(s) will be imported/transported within the United States in accordance with the terms and conditions as are specified in the permit. The Permittee is the legal importer/recipient [as applicable] of regulated article(s) and is responsible for complying with the permit conditions. The Permittee must be at least 18 years of age and have and maintain an address in the United States that is specified on the permit; or if another legal entity, maintain an address or business office in the United States with a designated individual for service of process; and serve as the contact for the purpose of communications associated with the import, transit, or transport of the regulated article(s). **Note: Import/Permit requirements are subject to change at any time during the duration of this permit.

- ***Each shipment shall be accompanied by an ORIGINAL signed document from the producer/manufacturer confirming that: **1)** the exported material was derived from wild boar (porcine) that originated in Japan; **2)** prior to export to the United States, the exported materials were treated as follows: **(a)** the teeth were treated using a solution of bleach in an ultrasonic cleansing system, **(b)** the hair samples were washed with isopropanol and air dried, **(c)** the slides contain blood fixed with methanol and acetic acid then sealed with a fixative and coverslip, and ...[continued on page 2]...

continued on subsequent page(s).....

TO EXPEDITE CLEARANCES AT THE PORT OF ENTRY, BILL OF LADING, AIRBILL OR OTHER DOCUMENTS ACCOMPANYING THE SHIPMENT SHALL BEAR THE PERMIT NUMBER

SIGNATURE Deborah Langford
DShangford

TITLE
Staff Veterinarian
National Import Export Services

NO. LABELS

VS FORM 16-6A (MAR 95) Replaces VS Form 16-3A and 16-28 which are obsolete

RESTRICTIONS AND PRECAUTIONS: (continued from Permit Form VS 16-6)

- ...[continued from page 1]... **(d)** the blood samples were fixed with methanol and acetic acid plus glutaraldehyde to achieve a final concentration of 0.2% glutaraldehyde; and **3)** the exported material was not exposed to or commingled with any other animal origin material.
  [This certification must CLEARLY correspond to the shipment by means of an invoice number or shipping marks or lot number or other identification method. An English translation must be provided.]

- COMMERCIAL DISTRIBUTION OF THE IMPORTED MATERIAL IS PROHIBITED.

- This permit DOES NOT authorize direct or indirect exposure of or inoculation into laboratory and domestic livestock (including but not limited to: birds/poultry, cattle, sheep, goats, swine, and/or horses). Work shall be limited to *in vitro* uses only. No extraction of nucleic acids is to be performed on imported material.

- Packaging, containers, and all equipment in contact with these materials shall be sterilized or considered a biohazard and be disposed of accordingly.

- THIS PERMIT IS VALID ONLY FOR WORK CONDUCTED OR DIRECTED BY YOU OR YOUR DESIGNEE IN YOUR PRESENT U.S. FACILITY OR APPROPRIATELY INSPECTED LABORATORY. THE AUTHORIZED IMPORTED MATERIAL(S) MUST BE SHIPPED/CONSIGNED DIRECTLY TO THE ADDRESS OF THE PERMITTEE OR TO THE ADDRESS OF THE ADDITIONAL PERMITTEE(S) AS IDENTIFIED ON THIS PERMIT. (MATERIALS SHALL NOT BE MOVED TO ANOTHER U.S. LOCATION, OR DISTRIBUTED WITHIN THE U.S., WITHOUT USDA, APHIS, VS, NIES AUTHORIZATION.)

- On completion of your work, all permitted materials and all derivatives therefrom shall be destroyed.

- This permit does not exempt the permittee from responsibility for compliance with any other applicable federal, state, or local laws and regulations.

- Imported material may be subject to regulations enforced by the United States Department of Interior, Fish and Wildlife Service (FWS). Importer must contact FWS, information is available at web pages http://www.FWS.gov/permits/ and/or http://www.FWS.gov/le/travelers.html

- The restrictions on this permit remain in force as long as the material is in the United States.

U.S.DEPARTMENT OF AGRICULTURE
APHIS / VETERINARY SERVICES, RIVERDALE, MARYLAND 20737.

ATTACH TO U.S. VETERINARY PERMIT - 132546

RESTRICTIONS AND PRECAUTIONS: (continued from Permit Form VS 16-6)

- Any person who VIOLATES the terms and conditions of permits, and/or who forge, counterfeit, or deface permits may be subject to criminal and civil penalties in accordance with applicable law. In addition, all current permits may be cancelled and future permit applications denied.

- A copy of this permit must be included with the shipping documents. For imported materials, these documents must be presented to CBP Agricultural Specialists upon arrival at the U.S. port of arrival.