RESEARCH REPORT
SWINE HEALTH

Title: Development and Evaluation of a Standardized Protocol for Utilization of Swine Oral fluids for PCR detection of High Consequence Pathogens: FMD, CSF, and ASF **- NPB #14-284 revised**

Investigator: Jessie D. Trujillo, DVM, PhD

Institution: Kansas State University

Date Submitted: 2/14/2018

Industry Summary:

The primary objective of this project was to develop a standard operating procedure for the use of swine oral fluids (SOF) as a diagnostic specimen for detection of endemic viral diseases such as Swine Influenza virus (SIV) and Porcine Reproductive Respiratory Disease virus (PRRSV) and foreign animal viral disease pathogens (FAD) such as African Swine Fever virus (ASFv), Classical Swine Fever virus (CSFv) and Foot and Mouth Disease (virus FMDv). For the execution of this research project viruses (BSL2 pathogens: SIV and PRRSV) and surrogate viral nucleic acids (NA) for ASFv. CSFv and FMDv) in varying concentrations were spiked into a collection of swine oral fluids to mimic what would appear in a clinical sample. Spiked SOF were then put through a battery of various extraction protocols followed by testing of clinical samples in BSL2/BSL3. NA recovery was tested using qPCR detection protocols based on regulatory protocols used by National Animal Health Laboratory Network (NAHLN) laboratories. Challenges arise since SOF are a variable biological specimen with regard to donor numbers (multiple pig contributors), saliva components, viscosity and host epithelial components. Additionally SOF are considered to be fecal, feed and environmental samples which are some of the most challenging diagnostic specimens for both human and animal medicine. These variables contributed to the failures of consistent recovery of low copy number pathogen NAs from SOF and the ability to amplify and detect pathogen RNA from this biological specimen following rapid magnetic bead extraction protocols. The presence of inhibitors existing in SOF only inhibit the ability to bind NA to extraction beads used but also inhibit PCR detection of pathogen NA. The good news is that for the vast majority of these inhibitors, their effects follow a linear scale (1X), thus a 2X or 4X dilution can result in most cases minimizing these inhibitors effects, while having minimal effect on PCR sensitivity (log-10X scale). Additionally, modifications to the bead protocol to help deal with this challenging diagnostic specimen greatly improve NA recovery. Finally, what appears to be an optimal protocol is to balance the dilution of SOF with a modified magnetic bead NA extraction protocol for reduction of PCR inhibitors, and not the addition of more SOF into the protocol. By do so, this leads to more favorable sensitivity and reproducibility with regard to quantitative qPCR detection of swine pathogens in SOF. Although the SOP for SOF preparation will need undergo specific end user validation such as the NAHLN, this research can provide boiler plate SOP for pathogen detection in SOF. JD Trujillo. Trudiagnostics@outlook.com.

Keywords: Swine oral fluids, Standard operating procedure, qPCR, swine viral pathogens, PRRSV, ASFV.

Project summary: The primary objective of this project is to develop and validate a standard operating procedure (SOP) for the optimal purification of pathogen NA and quantitative PCR detection of high consequence Swine pathogens from swine oral fluids (SOF). This study employed the comparative use of three commercially available magnetic bead extraction kits (GeneReach, USA; Qiagen, Germany; and Life Technology, USA) and various SOF samples. Nucleic acid surrogates (provided by FADDL) for Foot and Mouth Disease (FMDv), Classical Swine Fever virus (CSFv) and African Swine Fever virus (ASFv) and live virus stocks were used for Influenza A (pH1N1), Porcine Reproductive and Respiratory Syndrome virus (PRRSV) and AFSV were utilized. Various concentrations of targets (surrogates, viruses) were spiked into SOF and extracted side by side using three automated extraction instruments (GeneReach Tacomini- 8 well, Qiagen BioSprint- 96 well and Life technology King Fisher 24 (BSL3)). Protocols utilized based on kit manufacture recommendations with experimental modifications included varying volume and dilution of SOF, varying kit components and using several pre-extraction treatments. Extractions performed in duplicate or triplicate, evaluated the effect of experimental parameters on the extraction efficiency via qPCR NA detection. PCR detection of Influenza A, FMDv, and CSFv were performed per National Veterinary Service Laboratory protocol utilizing two RT (reverse transcriptase)-qPCR master mixes (Life technologies-NVSL SOP reagents and Quanta Biosciences, USA, alternate). Quanta Biosciences qPCR mix (Fast mix II) was utilized for the detection of ASFv DNA (modified NVSL protocol). Detection of PRRSv RNA was performed using the Qiagen Virotype PRRSv kit. PCR was performed with the CFX 96 qPCR machine (Biorad, USA). Clinical samples tested with the developed protocol were SOF containing PRRSV and SIV positive and various previous qPCR tested negative samples (ISU VDL). For BSL 3 work, oral fluids collected from swine experimentally infected with Armenia 2007 strain of AFSV (BSL3 BRI-KSU) were used.

Results: GeneReach and Qiagen magnetic bead extraction kits yielded nearly equivalent nucleic acid (NA) recovery for all five targets with greater than 90-99% recovery of NA utilizing 100 200ul of SOF or 200 ul of diluted (1:2 or 1:4) SOF in the extraction. Continued issues with magnetic beads used in the Applied Biosystems mixes greatly hindered our ability to attain reliable and reproducible data using these reagents and thus they were not investigated using clinical samples in either BSL2 or BSL3. In contrast, use of 300ul of even diluted (1:2) SOF and some cases 200 ul of SOF into the extraction resulted in markedly reduced recovery (0-45%) with high variability of PCR determined copy number for qPCR targets particularly when live virus or clinical samples were used. Application of the test protocols using low volume or diluted and clarified SOF improved sensitivity of detection using clinical samples. Application of this protocol at BSL3 for the detection of ASFV in SOF resulted in reliable detection of ASFV in a pen of 5 infected pigs one day prior or on the same day ASFV DNA was detected in the blood and often 3-4 days prior to the onset of clinical disease.

Conclusion: Studies demonstrate that optimal volume and dilution of SOF utilized and the amount of elution buffer and magnetic beads when using magnetic bead extraction is critical for reproducible and sensitive detection of live virus and/or low copy pathogen NA. This work provides candidate a SOP for consideration of use and alternate PCR reagents for FAD surveillance using swine oral fluids.

Introduction:

Accurate, real-time pathogen detection is necessary for optimal animal health in the commercial swine industry, and will provide immediate detection of the incursion of foreign animal diseases such as FMDv, CSFv and ASFv in to the US. The recent introduction of Porcine Endemic Diarrhea virus into the US swine population emphasizes the urgent need for continuous and diligent surveillance for FAD in swine. Cost and effort associated with traditional diagnostic sampling methods (blood, feces, or nasal swab) from individual pigs are primary barriers to accomplishing this goal. Oral fluid sampling is much less laborious, less costly and nearly stress free for the collectors and the swine. The use of oral fluid specimens is essentially changing the face of infectious disease surveillance for endemic diseases in U.S. swine population and could greatly alter the ability to detect foreign or emergent pathogens as well.

Swine oral fluids testing have become a very common means of testing swine for the presence of pathogen nucleic acids. However, testing results demonstrate that swine oral fluid testing can provide quite variable results when the probability of detection is compared across multiple diagnostic laboratories utilizing similar yet different sample preparation methods and molecular based detection methods; particularly in situations with low disease prevalence or low to moderate pathogen are present; the prepatent period (preclinical). Unfortunately this is the situation when surveillance diagnostics is most powerful in its ability to mitigate the impacts of a pathogen. Thus, the main objective of this work was to develop a standardized protocol for the optimized extraction of viral nucleic acids from swine oral fluids for molecular based detection of high consequence pathogens. The development of a standardized protocol which can then be optimized, validated and utilized for specific pathogen detection methods by a particular end user for the determined fit for purpose.

By doing so this will allow SOF to become a more reliable and consistent specimen for pathogen detection within a population, quite early in infection when pathogen prevalence is at its lowest and when the biggest negative impact of agent incursion can be mitigated.

Objectives: The primary objective of this NPB project is to develop a standardized protocol for optimized purification of viral nucleic acids from swine oral fluids for molecular based detection of high consequence pathogens utilizing NAHLN quantitative qPCR protocols.

Materials & Methods:
This study employed the comparative use of three commercially available magnetic bead extraction kits (GeneReach, USA; Qiagen, Germany; and Life Technology, USA) and various SOF samples. Nucleic acid surrogates were used for Foot and Mouth Disease (FMDv), Classical Swine Fever virus (CSFv) and African Swine Fever virus (ASFv) and live virus stocks were used for Influenza A (pH1N1), Porcine Reproductive and Respiratory Syndrome virus, and ASFV. Varying concentrations surrogate nucleic acids or virus were spiked into clarified SOF samples of varying consistency and characteristics. Experiments were performed in duplicate or triplicate. Various volumes of pretreated and/or diluted spiked SOF were extracted side by side using three automated extraction instruments (GeneReach Tacomini- 8 well, Qiagen BioSprint- 96 well, and Life technologies 24 well King Fisher (BSL3)). Protocols utilized were based on kit manufacture recommendations with experimental modifications including varying volume and dilutions of oral fluids, varying kit components and or volumes and employing several pre-extraction treatments. Extractions were performed in duplicate or triplicate, to evaluate the effect of input volume or dilution of SOF on the extraction efficiency or nucleic acid detection for protocol optimization. Clinical samples tested with the protocol were SOF containing PRRSV and SIV collected at the ISU VDL, PCR negative SOF samples, and oral fluids collected from swine experimentally infected with ASFV Armenia 2007 (BSL3 BRI-KSU). Real time PCR detection of Influenza A, FMDv, and CSFv were performed per National Veterinary Service Laboratory protocol utilizing two commercially available RT-qPCR master mixes (Life technologies-NVSL SOP reagents and Quanta Biosciences, USA). Quanta Biosciences qPCR master mix (Fast mix II) was utilized for the detection of ASFv DNA. Detection of PRRSv RNA was performed using the Qiagen Virotype PRRSv kit. PCR was performed with the CFX 96 qPCR machine (Biorad, USA).

Results:
The primary solution to the problem is dilution and more is not better in the case of using SOF as a biological specimen in magnetic bead extraction protocols. However, more is better with regard to certain components of the bead extraction. Using the developed protocol, GeneReach and Qiagen magnetic bead extraction kits yielded nearly equivalent NA detection for all five targets with greater than 90-99% recovery of NA utilizing 100-200ul of SOF or 200 ul of diluted (1:2 or 1:4) SOF in the extraction. In contrast, use of 300ul of even diluted (1:2) SOF into the extraction resulted in markedly reduced recovery (0-45%) of pathogen NA with high variability of PCR determined copy number for all targets particularly when live virus or clinical samples were used, pretreatment with heat only slightly improved virus recovery. Clarification of SOF via low spend centrifugation is a necessary pretreatment. Results were independent of extraction instrumentation or PCR assays. However, the use of isopropanol vs ethanol in the extraction protocol had modest impact on particularly DNA and lesser on RNA recovery when using beads as a binding substrate. Application of the SOF SOP using low volume (<200 ul) or diluted SOF resulted in improved sensitivity of detection using clinical samples (PRRSV and SIV). Also of interest and although not investigated experimentally here, it was observed that freezing and thawing the swine oral fluids even for brief periods improved pathogen detection by PCR methods. Perhaps this is due to freeze inactivation/denaturation of currently unknown proteins that aggregate pathogens and/or inhibit the binding of NA to the magnetic beads. It is unfortunate that continued issues with magnetic beads used in the Applied Biosystems extraction kits particularly when using two of the three magnetic bead extraction machines (Qiagen and GeneReach) greatly hindered our ability to attain reliable and reproducible data using these reagents and thus they were not investigated using clinical samples in either BSL2 or BSL3.

BSL3 work for the detection of ASFV in SOF resulted in reliable detection of ASFV.in a pen of 5 infected pigs one day prior or on the same day ASFV DNA was detected in the blood 3-4 days prior to the onset of overt clinical disease.

Discussion:

With this work, we are able to demonstrate that the use of larger volumes (>200ul) of clarified undiluted or even diluted SOF was markedly detrimental on the recovery and detection of low to moderate concentrations of pathogen or pathogen nucleic acids present or spiked into swine oral fluids when using SOF optimized magnetic bead extraction protocol using commercially available magnetic bead extractions kits and automated bead processors. To our knowledge this is this first study to investigate specimen volume and dilution and various magnetic bead extraction reagents with optimized protocols for swine oral fluids. Moreover, this study utilized the detection of 5 swine pathogens using four NAHLN approved molecular detection protocols and one commercially available reagent set (no NAHLN PRRSV qPCR SOP) as investigational endpoints.

Unfortunately, we were unable to test protocol performance in BSL3 containment using swine oral fluids containing FMDV and CSFV due to delays in receiving clearance to work in BSL3 containment facility outside of Manhattan, Kansas. However, data attained from the use of the SOF optimized extraction protocol with the two endemic RNA pathogens, Influenza A and PRRSV and USDA FMDV, ASFV and CSFV NA surrogates will provide strong data sets of consideration of this protocol use for the detection RNA vesiculoviruses such as FMDV, CSFV, Seneca Valley Virus and several others. Testing of swine oral fluids at BSL3(KSU-BRI) collected daily from pig experimentally infected with ASFv did demonstrate the practicality and feasibility of this protocol for the rapid detection of ASFv present in SOF in some cases prior to the onset of virus detection on blood using the same extraction kits and quantitative PCR detection protocol. Testing for the detection of Seneca Valley Virus and Vesicular Stomatitis Virus using this SOF SOP with recently developed highly sensitive quantitative qPCR assays maybe of future interest to the NPB.

Project data are being completed into a manuscript (Optimized Automated Magnetic Bead Extraction Protocol for Sensitive Real time PCR Detection of High Consequence Pathogens in Swine Oral Fuids., Trujillo et., al, in preparation) that will be published in a relevant peer review journal as well as a data dossier that will be presented to the NAHLN working group for consideration of this method for valuation and use for FAD surveillance in the US. Moreover, this protocol is being field tested in the DHS Point of Need diagnostic project being executed by the PI. This project will include protocol translation to collaborating global partners in regions of the world where FAD are endemic and thus might help mitigate the risk of incursion into the US. Field tested data with strengthen data sets to be presented to the NAHLN working group. Lastly, work with endemic pathogens such as SIV and PRRSV provide science base evidence of protocol utility which will be published in upcoming peer review journal. Additionally, evaluation of the protocol for use in Vesicular Stomatitis virus and Seneca Valley Virus detection oral fluids would also broaden project impacts with regard to US swine producers. Preliminary data was presented at the PRRSV meeting in Chicago 2014 and the subset of ASFv oral fluid data was presented at CRWAD in December 2017.

References related to this project:

1. Optimized Automated Magnetic Bead Extraction Protocol for Sensitive Real time PCR Detection of High Consequence Pathogens in Swine Oral Fluids., **JDTrujillo** et., al, in preparation.

2. Point of Need Molecular Based Detection of African Swine Fever Virus., **J.D. Trujillo**, K. Urbaniak, T. Wang, I. Morozov, J. A. Richt, Oral presentation., Conference of laboratory Animal Workers, Chicago, December 2017.

3. Point of Need Molecular Based Detection of African Swine Fever Virus., **J.D. Trujillo**, K. Urbaniak, T. Wang, I. Morozov, J. A. Richt, Poster presentation., PRRSV/FAD, Chicago, December 2017.

4. Development of candidate Standard Operating Procedures for rapid and sensitive molecular surveillance of Foot and Mouth Disease (FMDv), Classical Swine Fever virus (CSFv) and African Swine Fever virus (ASFv) utilizing swine oral fluids. **Jessie D. Trujillo**, Igor Morozov, Luis Gimenez-Lirola, Jeff Zimmerman, poster presentation, PRRSV/FAD meeting, Chicago December 2015.

5. Industry and academic collaborations for maintenance of the virotype® PRRSV RT-PCR reagents for reliable pathogen detection: Continual employment of scientific tools are essential. **Trujillo, JD** et al., poster presentation. North American PRRSv symposium, Chicago, IL December 2014.

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