RESEARCH REPORT
SWINE HEALTH

**Title:** Improving the performance of PRRSV oral fluid diagnostics – **NPB #15-158**

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**Industry Summary:**

It is widely accepted that PRRSV can be monitored in swine populations more conveniently, efficiently, and cheaply using oral fluid specimens as compared to surveillance based on testing individual pig serum samples. Oral fluids are a convenient sample to collect, but are often heavily contaminated with feed, feces, and inorganic environmental debris. Attempts to "clean up" samples by centrifugation or filtration have not been effective.
Alternatively, we have identified a coagulant formulation among several evaluated that is compatible (do not interfere/inhibit test performance) with antibody and PCR-based testing. Preliminary results indicate that this formulation could also assist in:

1. **Cleaning-up:** removing particulates from oral fluid samples (Figure 2).
2. **Sample handling:** improving the "handling characteristics" (pipetting) of the sample.
3. **Antibody detection:**

- Reducing ELISA non-specific reactions (background).
- Improve or retain the PRRS antibody ELISA sample-to-positive (S/P) response of antibody-positive samples compared to non-treated oral fluid samples → do not affect antibody-based tests performance.

4. **Nucleic acid (RNA) detection:**

- Selected coagulant is “PCR friendly” and did not cause PCR-inhibition at its optimal concentration.
- Selected active formula precipitated out of the matrix, suggesting the possibility of concentration of PCR targets by precipitation, thereby improving the sensitivity of PRRSV RT-PCR assays.
- Current research is investigating the possibility of improving oral fluid PCR diagnostics by concentrating targets in the oral fluid precipitate.

5. **Field application:** lyophilized format is ready for use in the field under ambient conditions → do not required.

The ultimate goal of this research line is to provide with more effective, efficient, inexpensive methods of surveillance for use in the prevention, control, and/or elimination of PRRSV and other economically-significant infectious agents.

**Keywords:** PRRSV, oral fluids, clean-up, antibody, ELISA, RNA, rRT-PCR.

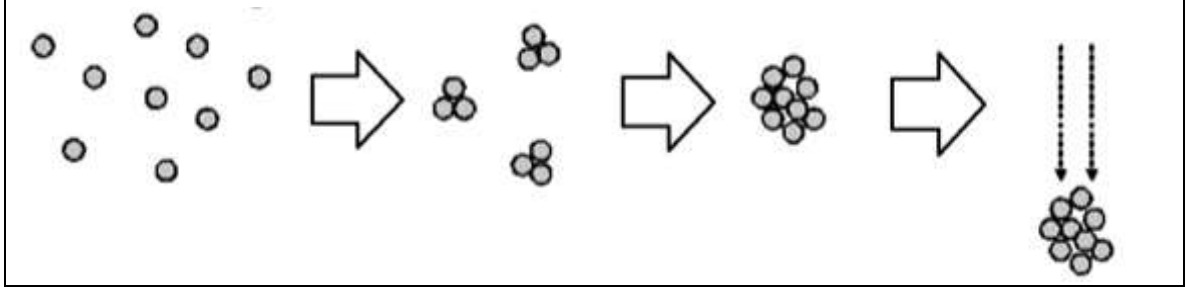
**Scientific Abstract:** This should be a scientific description limited to one page in length to describe your project and its results.

**Introduction:**

The purpose of surveillance is to guide efforts to control infectious disease, assure animal health and welfare, and improve producer profitability. Although serum is the traditional ante mortem surveillance sample, oral fluid is recognized as a more efficient and cost-effective alternative specimen. However, oral fluids are often contaminated with feed, feces, and inorganic particles from the environment, which in the severest cases, may affect test performance. Removal of particulates by centrifugation or filtration is not a solution because centrifugation removes diagnostic targets, filtration is too expensive, and both procedures require too much time to be performed routinely in the diagnostic laboratory.

This project is was a continuation of our work on the improvement of oral fluid diagnostics by treating samples with "coagulants". Coagulants are nonhazardous chemicals that cause particles to destabilize, clump together, and then precipitate out of solution (Figure 1). Coagulants have a long history of use in a wide variety of applications, including the treatment of drinking water, wastewater, and swimming pool maintenance. Their mode of action is conceptually simple, very fast, and highly effective at removing solids from liquids.

**Figure 1. "Clean up" based on the process of coagulation - flocculation - precipitation**



| Particles produce turbidity | Coagulation | Flocculation | Precipitation |
|---|---|---|---|

**Objectives:**

The objective of this project is to create an inexpensive and rapid oral fluid “clean up” protocol that improves both PRRSV oral fluid ELISA and RT-PCR diagnostic performance in high-throughput laboratories. In order to achieve this objective, we followed the next experimental design:

A. Generate a panel of oral fluid samples from pigs of known PRRSV status.
B. Use samples of known PRRSV status to optimize the clean-up procedure.
C. Evaluate the effect of coagulant treatment on PRRSV antibody detection in experimental samples.
D. Improve the performance of the PRRSV RT-PCR for oral fluid.

**Materials & Methods:**

**A. Establish a panel of oral fluid samples from pigs of known PRRSV status.**

- Twelve pigs (70 to 90 lb) were inoculated with a PRRSV modified-live vaccine (MLV) and oral fluid samples will be collected from individual pigs to develop samples of precisely known status.

- Individual pig oral fluid samples were collected daily from DPV -7 throughout the end of the study. Serum samples were collected on day post vaccination (DPV) -7, 0, 4, 6, 8, 10, 12, 14, 17, 21, 28, and 35.
- Serum samples were tested by PRRSV RT-PCR and PRRS ELISA to confirm animal status. This approach has been extremely effective in early related research ("BIVI 2014 Advanced PRRS Research") in detecting slight, but significant, differences among treatments.

**B. Use samples of known status (Part A) to optimize the cleanup procedure.**

- Establish the optimal coagulant concentration for use in oral fluids.
- Validate coagulant-based cleanup formulation and protocol for high throughput sample processing.
- Evaluate compounds (salts, hydrophilic polymers, detergents and/or organic solvents) known to synergize with coagulant-based precipitation.

**C. Evaluate the effect of coagulant treatment on PRRSV antibody detection in experimental samples.**

- Verify the improved performance (analytical sensitivity and repeatability) of the PRRSV oral fluid ELISA.
- Sample aliquoting: one aliquot were tested using the current procedure (no treatment) and one aliquot will be treated with coagulants and then tested simultaneously with its pair. Samples were tested in triplicate in order to measure within-sample test variation (repeatability).
- Categorical (Pos/Neg) and quantitative (ELISA S/P ratios) were analyzed for treatment effects. Repeatability coefficients were calculated for both untreated and treated samples.

**D. Improve the performance of the PRRSV RT-PCR for oral fluid.**

- We evaluated different viral RNA extraction/concentration protocols after oral fluid clean up step: 1) explored different viral RNA precipitation protocols from the supernatant; 2) explored different pellet handling and viral RNA extraction protocols.

**Results:**

**A. Establish a panel of oral fluid samples from pigs of known PRRSV status.**

- Oral fluid collection: Oral fluid samples were collected from each animal by hanging a cotton rope to be chewed for 20-30 minutes *(Kittawornrat et al. 2010)*. The fluid was extracted by wringing the wet portion of the non-treated rope in an individual plastic bag (Seal-Top Bag 10x12) and was drained into a 50ml centrifuge tube (Falcon™ 50ml). A total of 600 oral fluid samples were collected sampling twice per day, beginning 7 days prior vaccination (DPV -7) and continuing through 42 days post vaccination (DPV 42). Every sample was split into six aliquots using 5ml Cryovial tubes and stored at -80C until be tested.

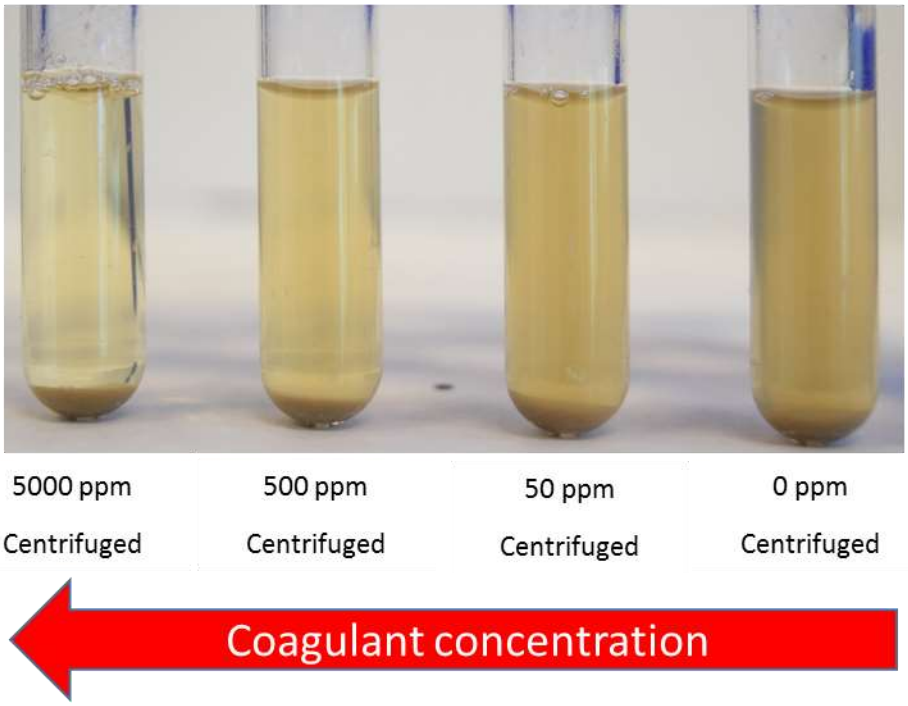
- Sera collection: Samples were collected to verify seroconversion to PRRSV. Serum samples were collected from each pig at DPV -7, 0, 14, 17, 21, 28, 35, and 42. We also collected serum from pigs 1-4 at DPV 3, 6 and 9; from pigs 5-8 at DPV 4, 7 and 10; and from pig 9-12 at DPV 5, 8 and 11. A total of 132 serum samples were collected, processed, aliquoted, and stored at -80C until use.

**B. Use samples of known status (Part A) to optimize the cleanup procedure.**

- Establish the optimal coagulant concentration for use in oral fluids. We evaluated different concentrations of the selected coagulant to stablish a working range for use in oral fluids. The

"cleaning power" (see turbidity in figure below) increased as coagulant concentration increased (ppm) compared to non-cleaning pretreatment (just centrifugation).

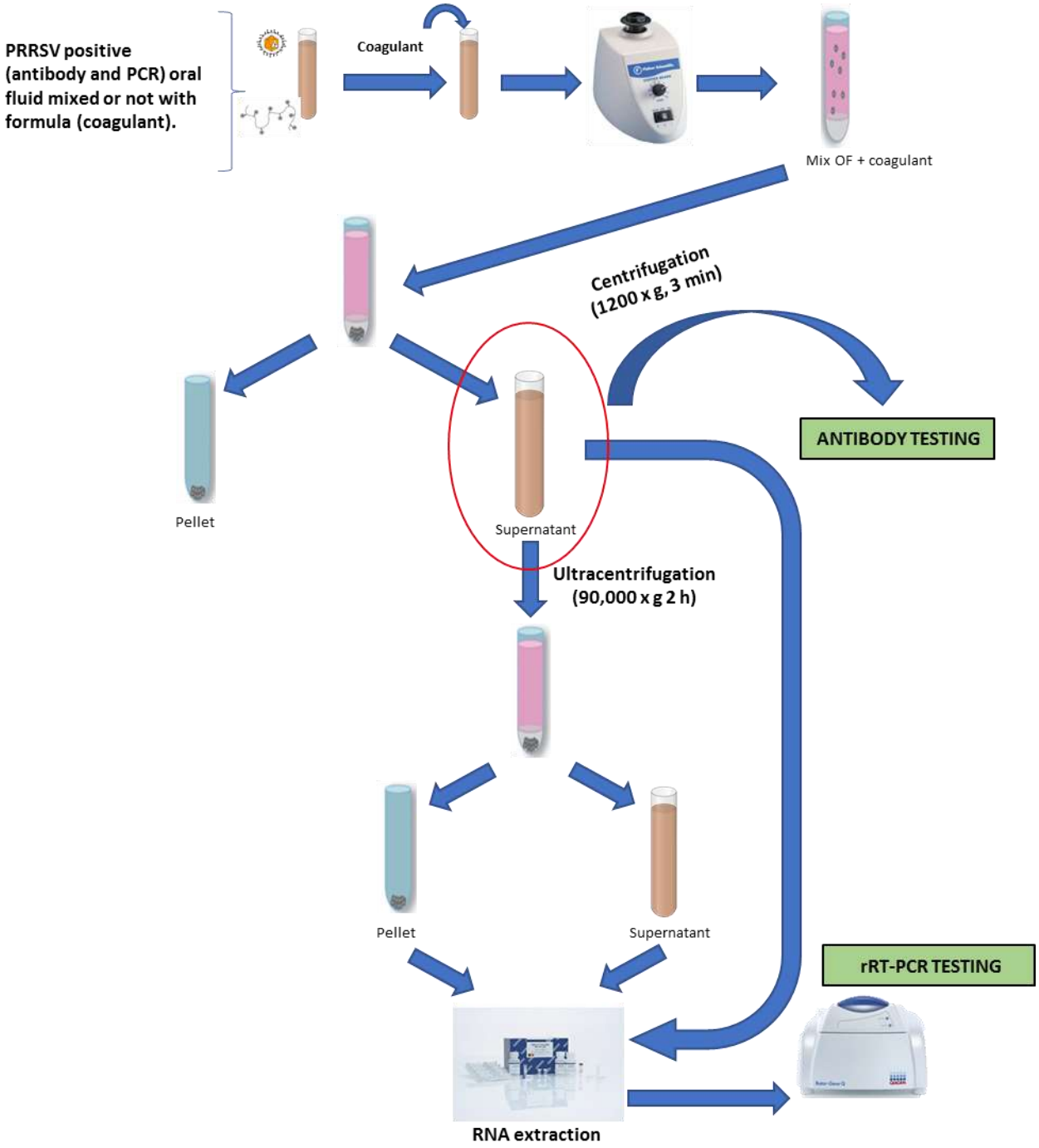
Figure 2. Cleaning effect resulted of oral fluid treatment with increased concentrations of coagulant




- The sample resulted from cleaning procedure with coagulant-based formula allow for high throughput sample processing (e.g., use of liquid handling robotic system).

**C. Evaluate the effect of coagulant treatment on PRRSV antibody detection in experimental samples.**

- Experimental samples were processed as follow:




- Oral fluid samples were analyzed with IDEXX PRRS Oral Fluids Ab Test (IDEXX, USA). The kit is able to detect PRRSV specific IgG antibodies against both type 1 and type 2.
- The results were expressed as sample-to-positive (S/P) ratios. Oral fluid samples that had as S/P value greater or equal than the cut off value (≥0.40) were considered positive for PRRSV specific IgG antibodies (red highlighted).

| | Non-treated oral fluid (control) | | | | | | | | | | | | Pre-treated oral fluid (coagulant formulation) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| DPV | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| -3 | 0.15 | 0.03 | 0.10 | 0.14 | 0.04 | 0.00 | 0.08 | 0.02 | 0.09 | 0.02 | 0.03 | 0.04 | 0.08 | 0.02 | 0.05 | 0.05 | 0.01 | -0.01 | 0.12 | -0.02 | 0.00 | -0.01 | -0.07 | -0.02 |
| 0 | 0.06 | -0.01 | 0.06 | 0.05 | 0.07 | 0.14 | 0.05 | 0.14 | 0.14 | 0.03 | 0.17 | 0.03 | 0.02 | -0.02 | -0.08 | 0.00 | 0.03 | 0.04 | -0.05 | -0.03 | 0.04 | -0.01 | 0.08 | -0.03 |
| 3 | 0.06 | 0.06 | 0.06 | 0.05 | 0.07 | 0.08 | 0.07 | 0.00 | 0.15 | 0.02 | 0.01 | 0.09 | -0.07 | 0.02 | 0.07 | 0.01 | 0.00 | 0.03 | -0.03 | 0.00 | 0.02 | -0.03 | 0.02 | 0.01 |
| 7 | 0.02 | 0.06 | 0.03 | 0.12 | 0.05 | 0.04 | 0.07 | 0.08 | 0.12 | -0.02 | 0.02 | -0.01 | 0.00 | -0.08 | 0.03 | -0.04 | 0.01 | 0.03 | 0.01 | 0.12 | 0.01 | 0.01 | 0.00 | 9.03 |
| 9 | 0.45 | 0.43 | 0.12 | 0.90 | 0.35 | 0.22 | 0.22 | 1.32 | 0.15 | 0.21 | 0.04 | 0.35 | 0.27 | 0.34 | 0.12 | 0.73 | 0.14 | 7.47 | 0.18 | 1.36 | 0.18 | 0.16 | 0.00 | 0.26 |
| 12 | 5.25 | 3.28 | 2.70 | 8.71 | 7.65 | 2.14 | 4.56 | 10.41 | 5.93 | 1.24 | 0.86 | 5.97 | 5.33 | 3.73 | 2.46 | 5.03 | 6.50 | 1.25 | 4.15 | 10.53 | 5.10 | 1.75 | 0.81 | 2.46 |
| 14 | 8.08 | 4.11 | 1.88 | 9.95 | 7.77 | 3.84 | 8.51 | 8.09 | 5.88 | 2.25 | 0.45 | 6.36 | 8.00 | 3.69 | 2.20 | 10.26 | 7.92 | 2.93 | 5.21 | 7.83 | 6.05 | 2.05 | 0.65 | 7.26 |
| 17 | 10.58 | 6.78 | 3.15 | 10.10 | 9.96 | 2.29 | 12.31 | 8.77 | 6.72 | 3.70 | 1.76 | 7.58 | 11.01 | 6.70 | 3.00 | 9.29 | 8.79 | 2.32 | 11.09 | 8.93 | 6.79 | 3.72 | 1.46 | 6.70 |
| 21 | 9.02 | 4.99 | 4.87 | 10.19 | 8.97 | 10.31 | 8.94 | 10.16 | 7.73 | 3.18 | 5.41 | 8.80 | 6.94 | 5.72 | 4.00 | 9.63 | 8.91 | 9.45 | 5.44 | 9.79 | 8.25 | 4.08 | 3.80 | 8.21 |
| 24 | 6.91 | 6.04 | 3.27 | 11.44 | 9.56 | 7.29 | 10.99 | 9.68 | 4.80 | 5.31 | 2.17 | 9.06 | 7.09 | 6.11 | 3.32 | 10.76 | 9.72 | 7.10 | 10.68 | 9.19 | 6.39 | 4.86 | 2.33 | 5.47 |
| 28 | 3.69 | 5.87 | 2.54 | 9.98 | 9.96 | 10.37 | 10.40 | 7.56 | 6.14 | 4.29 | 4.39 | 9.33 | 3.86 | 6.33 | 2.34 | 9.29 | 9.76 | 9.36 | 10.21 | 6.52 | 6.20 | 4.34 | 3.99 | 9.91 |
| 31 | 7.83 | 4.33 | 3.97 | 10.19 | 9.56 | 10.18 | 9.03 | 7.59 | 6.22 | 1.85 | 1.66 | 9.56 | 8.77 | 4.39 | 4.13 | 9.79 | 7.36 | 9.49 | 9.28 | 3.22 | 4.99 | 1.92 | 1.71 | 8.19 |
| 35 | 8.28 | 3.62 | 3.07 | 11.98 | 11.35 | 6.82 | 6.84 | 6.58 | 4.10 | 2.41 | 2.62 | 6.68 | 6.00 | 1.36 | 3.16 | 11.59 | 10.22 | 7.00 | 7.79 | 7.08 | 4.24 | 1.18 | 2.16 | 5.99 |
| 38 | 6.61 | 4.68 | 5.33 | 9.56 | 9.74 | 8.52 | 9.20 | 6.71 | 3.29 | 2.43 | 2.12 | 7.19 | 6.95 | 4.69 | 5.19 | 6.99 | 10.03 | 8.93 | 9.56 | 7.17 | 4.02 | 3.51 | 2.05 | 7.25 |
| 42 | 6.75 | 3.52 | 4.02 | 11.44 | 10.26 | 4.11 | 9.40 | 5.69 | 3.25 | 1.37 | 3.91 | 5.84 | 7.57 | 3.64 | 3.84 | 10.76 | 9.15 | 4.44 | 7.54 | 2.73 | 3.50 | 1.19 | 3.43 | 2.45 |

- The ELISA test results showed non-statistical difference between non-treated vs treated (coagulant formulation) oral fluid samples through time. Oral fluid samples were as stable as non-treated samples when kept overtime (14 days) at 4C.

**D. Improve the performance of the PRRSV RT-PCR for oral fluid.**

- Precipitation/concentration of virus cannot be achieved by simply increasing the concentration of coagulant because this causes loss of antibody.
- An unanticipated effect of coagulant treatment was a reduction in the detection of PRRSV in the supernatant by RT-PCR as compared to untreated controls ($p < 0.001$). That is, coagulant removed PRRSV by precipitation, just as it removed other particulates from the oral fluid matrix.
- While unanticipated, the precipitation of PRRSV with coagulant treatment suggested an opportunity to concentrate PCR targets by precipitation and then testing the nucleic acid or virus-enriched precipitate.
- So far, we have been able to concentrate targets (virus) and increase PCR detection rate by combining precipitation (clean-up) by coagulation + ultracentrifugation.
- In addition, we are currently evaluating different viral RNA extraction/concentration protocols after oral fluid clean up step. Specifically, we are exploring different viral RNA precipitation protocols from the supernatant; 2) exploring different pellet handling and viral RNA extraction protocols.

**Discussion:**

Oral fluids are a convenient sample to collect, but are often heavily contaminated with feed, feces, and inorganic environmental debris. These characteristics make them unpopular with laboratory technicians and may affect test performance. Our research demonstrate that oral fluid-based surveillance can be improved upon by further developments in testing methods and procedures. Coagulants are nonhazardous chemicals that cause particles to destabilize, clump together, and then precipitate out of solution. We have developed an inexpensive and rapid oral fluid “clean up” protocol for the removal of the fine particles suspended in oral fluids using coagulant treatment. We have demonstrated that the removal of "debris" from oral fluid specimens (1) improve their acceptance by laboratory laboratory personnel, 2) it is compatible to

liquid-handling robots; (3) does not interfere with performance PRRSV antibody ELISA test of diagnostic assays; and (4) does not affect oral fluid stability over time (when stored at 4C after treatment) compared to untreated oral fluid samples.

An unanticipated effect of coagulant treatment was a reduction in the detection of PRRSV in the supernatant by RT-PCR as compared to untreated controls ($p < 0.001$). That is, coagulant removed PRRSV by precipitation, just as it removed other particulates from the oral fluid matrix. We are exploring the effect of PRRSV precipitation after treatment with coagulants in order to concentrate PCR targets (virus and nucleic acids) and improve the diagnostic and analytical sensitivity of oral fluid PCR assays for a variety of swine pathogens - not just PRRSV.

Ultimately, the value of this research to the pork industry is to further improve the current surveillance/diagnostic methods based on the use of oral fluids as alternative specimen for a more effective, efficient, inexpensive methods of surveillance for use in the prevention, control, and/or elimination of PRRSV and other economically-significant infectious agents.