NPB FINAL RESEARCH GRANT REPORT

NPB Project Title:	Oral fluid sample size for FAD detection at low prevalence in large commercial pigs NPB #19-067
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Industry Summary

Background Surveillance based on the detection of pathogen-specific antibody or nucleic acid in oral fluid samples is practical, cost-effective, diagnostically sensitive, and widely used by pork producers (Figure 1). This approach has been shown to be highly effective for the surveillance of both endemic and exotic pathogens, including African swine fever virus (Gimenez-Lirola et al., 2016), classical swine fever virus (Panyasing et al., 2018), foot-and-mouth disease virus (Senthilkumaran et al., 2017), and many others (Bjustrom Kraft et al., 2018; Henao-Diaz et al., 2020). For these reasons, the 2017 joint meeting of representatives of the National Pork Board, Swine Health Information Center, the Institute for Infectious Animal Diseases, and the Department of Homeland Security Center of Excellence concluded that oral-fluid sampling was vital to US Foreign Animal Disease (FAD) surveillance.

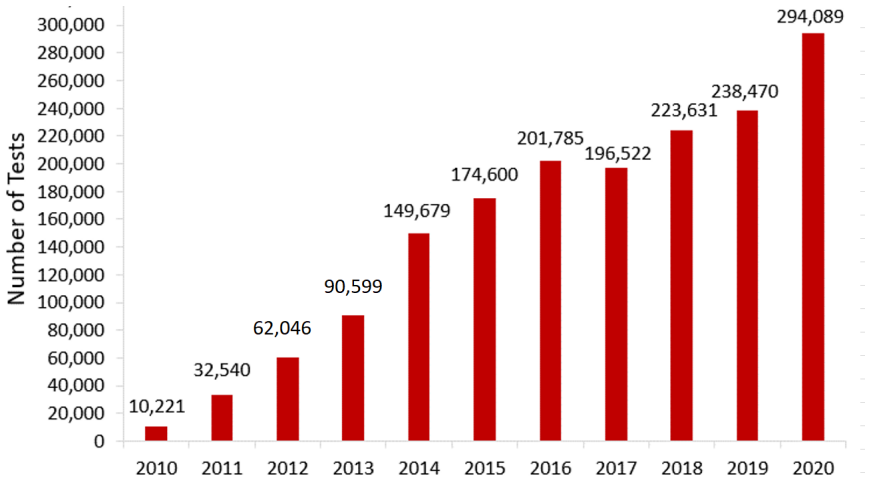

Figure 1. Number of swine oral fluid diagnostic tests performed at the Iowa State University Veterinary Diagnostic Laboratory by calendar year.

Statement of the problem Oral fluid sampling is possible because it is consistent with normal pig normal behavior. That is, pigs are curious and readily explore new objects in their environment by biting and chewing, thereby providing an oral fluid sample. In addition, pigs are highly social and, if one member of the group interacts with the rope, others will follow suit.

In terms of surveillance for endemic or exotic pathogens, the inevitable question is, "How many samples should I collect?" The vast majority of research on this question has focused on oral fluid sampling in pens of ~25 pigs. This work has shown that one rope will provide an oral fluid sample representing ~80% of the animals in a pen holding 25 pigs in a 30-minute sampling period (Pol et al., 2017; White et al., 2014). However, there is no "industry standard" and pens are generally evolving toward larger sizes.

To optimally sample swine populations for FAD detection, we need guidelines for oral fluid sampling across the range of pen sizes in modern production systems. Therefore, the objective of this study was to determine how samples should be collected from pens of various sizes, e.g., 20, 40, 60, 100, etc., in order to obtain a specimen that represents the infection status of the pigs in the pen. It is important to note that this project did not involve test performance assessment. Test performance is pathogen- and assay-specific; in contrast, sampling guidelines should be universal.

Research approach Earlier research on oral fluid sampling used infectious agents and/or modified live vaccines to model detection. This approach was highly effective and allowed for calculating the probability of detection (antibody or nucleic acid) as a function of within-pen prevalence (Olsen et al., 2013). However, using infectious agents in commercial herds is impractical and entails the risk of litigation. As an alternative, we evaluated sampling in the field using two measures: (1) detection of a safe, non-infectious surrogate (flunixin meglumine, FM) in oral fluid from a subset of FM-inoculated pigs, and (2) pig behavior, i.e., counts of the interactions of FM-inoculated pigs with ropes hung in the pen. FM has been FDA-approved for use in market swine since 2006 and has a withdrawal period of 12 days (Burr et al., 2006).

Key Findings

The study was conducted in 72 pens holding 21 to 98 pigs by hanging 4 ropes in each pen for 20 minutes, observing pig behavior, and then collecting oral fluids for FM analysis. Depending on pen size, each pen had 3 to 12 FM-inoculated pigs, i.e., "prevalence" was held relatively constant and pen size was varied. Analysis of pig behavior data showed that, on average, 79% of the FM-inoculated animals interacted with one or more ropes hung in the pens in a 20-minute observation period (Figure 2). In 70 of 72 pens (97.2%), ≥ 50% of injected pigs interacted with the rope. The 2 pens in which < 50% of the pigs interacted with a rope were smaller pens (≤ 25 head).

1. Given a fixed "prevalence", i.e., FM-inoculated pigs, pen size had no effect ($p = 0.54$, logistic regression) on the proportion of FM pigs that interacted with the rope. Based on these data, one rope is sufficient for detection in quadrangular pens holding up to 100 animals.

   NOTE: These results were surprising and counter intuitive. That is, our prior assumption was that detection would be more difficult in larger pens; such was not the case.

2. This study was done in simple quadrangular pens holding <100 pigs. Whether pens with more complex designs and holding > 100 pigs conform to these observations is not known but could be easily determined using the methodology described herein. Future work on oral fluid sampling should focus on pens holding more than 100 animals.

Keywords

Oral fluids, surveillance, sampling, detection, pig behavior, banamine, flunixin meglumine

Scientific Abstract

For surveillance, the inevitable question is, "How many samples must I collect for detection?"

The vast majority of research on this question has focused on oral fluid sampling in pens of ~25 pigs. This work has shown that one rope will provide an oral fluid sample representing ~80% of the animals in a pen holding 25 pigs in a 30-minute sampling period (Pol et al., 2017; White et al., 2014). However, there is no "industry standard" pen size and the industry is generally evolving toward larger pen sizes. To optimally sample swine populations for FAD detection, we need guidelines for oral fluid sampling across the range of pen sizes coming into modern production systems.

The objective of this study was to develop oral fluid sampling guidelines for contemporary pens sizes. Specifically, the goal was to determine how samples should be collected from pens of various sizes, e.g., 20, 40, 60, 100, etc., in order to obtain a specimen that represents the infection status of the pigs in the pen. It is important to note that this project did not involve test performance assessment. Test performance is pathogen- and assay-specific; in contrast, sampling guidelines should be universal.

Research approach Prior research on oral fluid sampling used infectious agents and/or modified live vaccines to model detection. This approach was highly effective, but the use of uncontrolled infectious agents in the field is impractical, hazardous, and entails legal risks. Therefore, we used two measures to evaluate sampling efficacy in this field study: (1) detection of a safe, non-infectious surrogate (flunixin meglumine, FM) in oral fluid from a subset of FM-inoculated pigs, and (2) pig behavior, i.e., counts of the interactions of FM-inoculated pigs with ropes hung in the pen. FM has been FDA-approved for use in market swine since 2006, with a withdrawal period of 12 days (Burr et al., 2006).

Key Findings

The study was conducted in 72 pens holding from 21 to 98 pigs by hanging 4 ropes in each pen for 20 minutes, observing pig behavior and then collecting oral fluids for FM analysis. Depending on pen size, each pen had 3 to 12 FM-inoculated pigs, i.e., "prevalence" was held relatively constant and pen size was varied.

Analysis of pig behavior data showed that between 33% and 100% of the FM-inoculated animals interacted with one or more ropes hung in the pens (mean 79%) in a 20-minute observation period. More specifically (Figure 2) in 70 of 72 pens (97.2%), ≥ 50% of injected pigs interacted with at least one rope. The 2 pens in which < 50% of the pigs interacted with a rope were smaller pens (≤ 25 head).

1. Given a fix "prevalence" (FM-inoculated pigs), logistic regression analysis showed that pen size had no effect (p = 0.54) on the proportion of FM pigs that interacted with the rope. These results were surprising and counter intuitive. That is, most had assumed that detection would be more difficult in larger pen; such was not the case.
2. This study was done in simple quadrangular pens holding <1 00 pigs. Whether pens with more complex designs and holding larger populations conform to these observations could be easily determined using the methodology described herein.

Introduction

This project addressed a NPB call for research on "*surveillance sampling methods for optimizing the detection of trade limiting foreign animal diseases within pens, barns, and sites".*

1. Diagnostic assays for nucleic acid and antibody detection in swine oral fluid specimens have been described for a variety of pathogens. More specific to the call and as reviewed by Henao-Diaz et al., 2020, the detection of ≥ 23 swine viral pathogens has been described (nucleic acid and/or antibody) in oral fluids, including African swine fever virus (Grau et al., 2015; Giménez-Lirola et al., 2016), classical swine fever virus (Dietze et al., 2017; Grau et al., 2015), FMDV (Senthilkumaran et al., 2017b), Japanese encephalitis virus (Lyons et al., 2018), Nipah virus (Kasloff et al., 2019), and swine vesicular disease virus (Senthilkumaran et al., 2017a). Thus, the feasibility of developing sensitive and specific assays for nucleic acid or antibody detection in oral fluids has been amply demonstrated.
2. Oral fluid-based surveillance for endemic pathogens has become routine in commercial herds in the U.S. For example, 295,000 oral fluid tests were performed at the Iowa State University Veterinary Diagnostic Laboratory in 2020 (R. Main, personal communication).
3. Oral fluid-based testing is recognized as highly diagnostically sensitive. Olsen et al. (2013) calculated the probability of PRRSV detection as a function of within-pen prevalence. As shown in Table 1, when within-pen prevalence was ≥ 15%, both RNA and antibody were easily detected in one pen-based oral fluid sample. In contrast, regardless of prevalence, multiple serum samples need to be collected from pigs in the pen to match the probability of detection achieved by one pen-based oral fluid sample (Table 1).

   Table 1. Probability of detecting PRRSV RNA (rRT-PCR) or antibody in pen-based oral fluids or individual pig serum samples (from Olsen et al., 2013)

	Probability of detection with one OF sample		No. of serum samples to match probability of detection with one pen-based OF sample	
Within-pen prevalence (%)	OF rRT-PCR	OF ELISA	Serum rRT-PCR	Serum ELISA
10	79%	59%	11	7
15	94%	85%	12	9
20	98%	94%	13	10
25	99%	97%	13	11

4. Current oral fluid sampling guidelines are based on field research conducted in pens of 25-30 pigs (Olsen et al., 2013; Rotolo et al., 2017; White et al., 2014). However, an informal survey conducted through the Iowa State University Department of Agricultural and Biosystems Engineering found that new swine facilities are being built with pens holding 22 to 500 animals. We do not have detection data to provide oral fluid sampling guidelines for these large pens sizes.

Objectives

The industry is evolving toward larger pen sizes. However, the research on oral fluid sampling has focused on pens of ~25 pigs. To optimally sample swine populations for FAD detection, we need guidelines for oral fluid sampling across the range of pen sizes coming into modern production systems.

Aim 1: Validate the use of flunixin meglumine for modeling oral fluid sampling in the field.

Aim 2: Develop rope sampling recommendation for a range of pen sizes in commercial production sites using the techniques developed in Aim 1.

Materials and methods

Aim 1. To validate the use of flunixin meglumine and to determine the timing and duration of rope sampling, individually housed pigs (n = 20; 37-58 kg) were administered flunixin meglumine (IM 2.2 mg/kg). To determine the pattern of excretion, oral fluid samples were collected at time 0 and between 16 minutes and 2 hours and 39 minutes post injection. Urine was collected from 15 of the 20 pigs utilizing free catch and tampon methods and the time of urination was recorded. Blood samples were collected via jugular venipuncture from the 20 pigs prior to flunixin administration and after final oral fluid collection (3 hours post injection).

To assess the potential for environmental contamination of ropes, 10 pigs (36-55 kg) were placed into the pens previously occupied by pigs that had received flunixin. Blood samples were collected from these pigs prior to being moved into the pens and following oral fluid sample collection. Oral fluids were collected prior to being placed into the individual pens. Ropes were immediately hung after being placed in the pen and collected between 24 minutes – 2 hours post placement. Environmental samples (Swiffer® wipes with phosphate buffered saline) were also collected from the pens prior to the administration of flunixin and following sampling of the non-injected pigs.

Aim 2. Four commercial swine facilities were used to develop rope sampling recommendations for a range of pen sizes. Barn design was consistent across sites: naturally ventilated, partially slatted flooring, and concrete and metal gating between pens. Pigs were commercial, cross-bred pigs and ranged in age from 10 – 13 weeks of age. All pigs were clinically normal at the time of the study.

The study was completed over two days at each facility. On day 1, pens were selected to minimize cross-contamination by skipping at least one pen between enrolled pens. If this was not possible, then adjacent pens were sampled simultaneously. In selected pens, oral fluid samples were collected using ropes hung in each of the four corners of each pen. The left corner closest to the alley was designated 1 each corner increased sequentially in a clockwise manner around then pen.

On day 1, a cotton rope was hung at position 1 in the pen for 20 minutes and tested to determine that there was no FM present in the pen. Pigs were also not administered FM prior to enrollment. The ropes were hung for 20 minutes and collected first into a sealable plastic bag and then poured into a 50 mL conical tube labeled with the pen number and date for identification. Samples were then transported in a cooler on ice to the ISU Field Services laboratory and split into two aliquots into 5 mL cryovial tubes and stored at -80 C until submission to the ISU Veterinary Diagnostic Laboratory.

On day 2, ~12% (median 90th percentile 9.8% - 15%) of pigs in each enrolled pen were injected with FM (Banamine-S, Merck) at the rate of 2.2 mg/kg (2 mL/100pounds) intramuscularly in the

cervical region. That is, depending on the number of pigs in a pen, either 3, 6, 10, or 12 pigs were injected with FM. These convenience-selected pigs were uniquely identified by writing a number on their back with livestock paint. Fifteen minutes after injection, ropes were hung at positions 1, 2, 3, and 4 for 20 minutes. During sample collection, the pen was monitored and a record maintained of the interaction of injected pig(s) with a rope using the number on the pigs' back for identification. After 20 minutes, oral fluid samples were collected by wringing the rope into a sealable plastic bag and then poured into a 50 mL conical tube with the pen and rope position number. To minimize the chance of sample contamination, gloves were changed between each rope. Samples were then transported in a cooler on ice to the ISU Field Services laboratory and split into two aliquots into 5 mL cryovial tubes and stored at -80 C until submission to the ISU Veterinary Diagnostic Laboratory for testing.

Following collection of oral fluids, a blood sample was collected from three FM-infected pigs in each pen. Pigs were conveniently selected for blood collection. Blood was collected via jugular venipuncture using a 16 gauge x 1.5 inch needle on a 12 mL syringe. The pig was restrained with a snare placed around the maxilla, and at least 2 mL of blood was collected from each pig. Blood was placed in a serum separator tube (BD, New Jersey) labeled with the pen and pig number for identification. Blood samples were transported in a cooler on ice to the ISU Field Services laboratory and split into two aliquots in 2.5 mL cryovial tubes and stored at -80 C until submission to the ISU VDL for testing, i.e., all testing for FM was done at the ISU VDL.

After blood collection, each pen was counted for the exact number of pigs it contained.

Serum testing. Serum samples were thawed and vortexed and 100 µL aliquots were transferred into a vial to which 400 µL of 250 ng/mL internal standard, flunixin D3 and 5-hydroxyflunixin D3 in acetonitrile with 0.1% formic acid was added. Standards were prepared by spiking 100 µL of blank serum with flunixin and 5-hydroxyflunixin to a concentration of 5, 10, 20, 50, 100, 200, 500, 1000, and 2000 ng/mL. Quality control (QC) samples were prepared by spiking 100 µL of blank serum with flunixin and 5-hydroxyflunixin to a concentration of 15, 150, and 1500 ng/mL. Samples were diluted by taking 25 µL of sample and diluting with 75 µL blank serum so ensure that the concentrations were on the standard curve. Results were then multiplied by 4 to account for this dilution. For several samples where the 5-hydroxyflunixin results were below the curve, the sample was re-extracted without dilution. Samples were vortexed and centrifuged at 2400 rpm for 20 minutes. Supernatant was transferred, and samples were dried down, reconstituted in 100 µL of 25% acetonitrile in water, vortexed and transferred to an autosampler vial (with glass insert), centrifuged for 20 min at 2400 rpm and analyzed via LC-MS/MS.

Serum concentrations of flunixin and 5-hydroxyflunixin were determined using UHPLC mass spectrometry. A Q Exactive Focus orbitrap was coupled to a Dionex Ultimate 3000 (Thermo Scientific, San Jose, CA, USA). The mobile phases consisted of A: 0.1% formic acid in water and B: 0.1% formic acid in methanol. The mobile phase began at 10% B with a linear gradient to 95% B at 0.5 min- 2 min. The gradient was maintained at 95% B for 1.75 min followed by re-equilibration to 10% B. The flow rate was maintained at 0.3 mL/min. A Hypersil Gold Vanquish column was used (50 mm x 2.1 mm, 1.9 µm particles) from Thermo (Thermo Scientific, San Jose, CA, USA) with the column temperature set to 40 °C. The injection volume was 2 µL for each. The following ions were used for identification and quantification: Flunixin (m/z 297.085) 264.050 and 279.074, Flunixin D3 (m/z 300.103) 267.050 and 279.074, 5-hydroxyflunixin (m/z 313.079) 280.045 and 295.069 and 5-hydroxyflunixin D3 (m/z 316.098)

280.045 and 298.088. The retention time for Flunixin and Flunixin D3 was 2.65, while the retention time for 5-hydroxyflunixin and 5-hydroxyflunixin D3 was 2.60.

Calibration curves were calculated using Quan Browser portion of the Xcalibur software and a linear fit. Both the Flunixin and 5-hydroxyflunixin calibration curve was 5-2000 ng/mL. The correlation coefficient (r^2) exceeded that of 0.99. The calibrators used were within a tolerance of ±15% of the nominal value except for the lower limit of quantification, which was < 20%. The QCs were within a tolerance of ±15% of the nominal value. The limit of detection (LOD) for flunixin was 0.3 ng/mL and the limit of quantitation (LOQ) which was based on the calibration curve was 5 ng/mL. The LOD for 5-hydroxyflunixin was 0.06 and the LOQ was 5 ng/mL.

Oral fluid testing. Oral fluid samples were thawed and inverted to mix and 1 mL aliquots were transferred into a glass tube. 100 ng internal standard, flunixin D3 in acetonitrile was added to blank, standards, and samples. Standards were prepared by spiking 1 mL of blank oral fluids with flunixin to a concentration of 5, 10, 20, 50, 100, 200, 500, 1000, and 2000 ng/mL. Quality control (QC) samples were prepared by spiking 1 mL of blank oral fluids with flunixin to a concentration of 15, 150, and 1500 ng/mL. One mL of citrate buffer at a pH of 3.3 was added and pH was adjusted to 2.9-3. Subsequently 5 mL of methyl tert-butyl ether was added to the tubes, which were then capped and mixed by inversion for 5 minutes. The tubes were then centrifuged at 2000 rpm for 20 minutes. The organic layer was transferred to a clean tube and dried down under nitrogen. The blank, standards, and samples were reconstituted with 100 µL of 25% acetonitrile in water, vortexed, and transferred to an autosampler vial (with glass insert), centrifuged for 20 min at 2400 rpm and analyzed via LC-MS/MS.

Oral fluid concentrations of flunixin were determined using UHPLC mass spectrometry. A Q Exactive Focus orbitrap was coupled to a Dionex Ultimate 3000 (Thermo Scientific, San Jose, CA, USA). The mobile phases consisted of A: 0.1% formic acid in water and B: 0.1% formic acid in methanol. The mobile phase began at 10% B with a linear gradient to 95% B at 0.5 min-2 min. The gradient was maintained at 95% B for 1.75 min followed by re-equilibration to 10% B. The flow rate was maintained at 0.3 mL/min. A Hypersil Gold Vanquish column was used (50 mm x 2.1 mm, 1.9 µm particles) from Thermo (Thermo Scientific, San Jose, CA, USA) with the column temperature set to 40 °C. The injection volume was 2 µL for each. The following ions were used for identification and quantification: Flunixin (m/z 297.085) 264.050 and 279.074, Flunixin D3 (m/z 300.103) 267.050 and 279.074. The retention time for Flunixin and Flunixin D3 was 2.65.

Calibration curves were calculated using Quan Browser portion of the Xcalibur software and a linear fit. Flunixin calibration curve was 5-2000 ng/mL. The correlation coefficient (r^2) exceeded that of 0.99. The calibrators used were within a tolerance of ±15% of the nominal value except for the lower limit of quantification, which was < 20%. The QCs were within a tolerance of ±15% of the nominal value. The limit of detection (LOD) for flunixin was 0.3 ng/mL and the limit of quantitation (LOQ) which was based on the calibration curve was 5 ng/mL.

Statistical Analysis. Statistical analyses were conducted using open-source software "R". P-values < 0.05 were considered to be statistically significant. The goal of the analysis was to establish the relationship between the probability that an individual pig housed in pen i interacted with the ropes (p_i) and its corresponding pen size (x_i). With y_{ij}, the binary response variable of whether a sampled pig j in pen i contacted with the ropes, a logistic regression was applied.

$$y_{ij} \sim Bernoulli(p_i) \quad (1)$$

$$\log\left(\frac{p_i}{1-p_i}\right) = \beta_0 + \beta_1 \cdot x_i \quad (2)$$

The logit transformed interacting probability p_i associates with the covariate x_i in a linear form, where the intercept β_0 represents the baseline, and the slope β_1 represents the changing rate of $\text{logit}(p_i)$ in response to different pen sizes.

Results

Aim 1: Flunixin meglumine was detected in oral fluids as quickly as 16 minutes post injection. These findings were consistent with a prior report by Coetzee (NPB #13-210). Flunixin and its metabolite were detected in urine as quickly at 1 hour and 31 minutes following injection. Environmental samples were all negative prior to the start of the study. All pens were positive for flunixin by environmental samples at the end of the study. Thus, FM could be detected in urine and could be transferred to untreated pigs, as previously reported by Hairgrove et al., 2019. That is, once in the environment, flunixin can be "picked up" and transferred to oral fluid samples.

These results supported the conclusion that FM could serve to model an infectious agent in field studies on oral fluids. Further, the results were used to establish sampling times for the field study conducted to achieve Aim 2.

Aim 2: The study was conducted in 72 pens holding from 21 to 98 pigs, each with 3 to 12 FM-inoculated pigs. FM was not detected in any samples collected on day 1, i.e., the pigs and their environment was free of FM. On day 2, among 288 ropes hung across all sites, 9 did not have a sufficient volume of oral fluid for testing leaving a total of 279 oral fluid samples. FM was detected in all serum samples and in 275 oral fluid samples. Among the 4 negative oral fluid samples, 3 were from pens of ~20 head and 1 from a pen of 88 head. Analysis of the concentration of FM in oral fluids showed no difference in concentration among samples as a function of pen size.

Analysis of pig behavior data showed that between 33% and 100% of the FM-inoculated animals interacted with one or more ropes (mean 79%) in a 20-minute observation period. More specifically (Figure 2) among the 72 pens, ≥ 50% of injected pigs interacted with at least one rope in 70 pens. The 2 pens in which < 50% of the pigs interacted with a rope were pen sizes of ≤ 25 head. Consistent with these observations, the logistic regression analysis showed that pen size had no effect ($p = 0.54$) on the proportion of FM pigs that interacted with the rope.

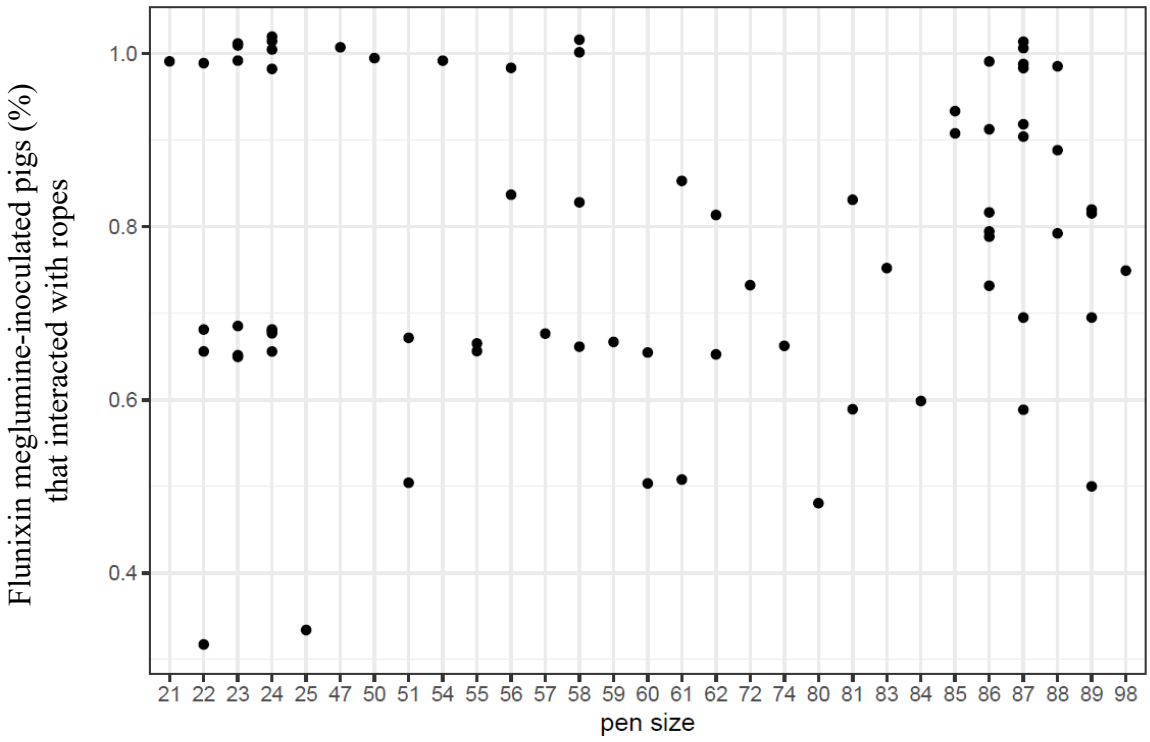

Figure 2. Percent of flunixin meglumine-inoculated pigs that interacted with one or more ropes (Y axis) over a period of 20 minutes by pen size (X axis). Given a fixed prevalence (~15%) logistic regression analysis showed that pen size had no effect on the probability that a FM-inoculated pigs would interact with rope ($p = 0.54$).

Discussion

1. Under the conditions of this study, i.e., pens holding between 21 and 98 pigs, the detection of a target at low prevalence was not more difficult in larger pens vs small. Thus, we conclude that one oral fluid sample is sufficient for detection in pens holding < 100 pigs.

   This observation was not anticipated by the investigators but can be explained as follows: in a pen of 20 pigs and a 10% prevalence (2 "target" pigs), one or both pigs can be distracted by other activities (eating, sleeping, interacting with pen mates) and not contribute to the oral fluid sample. In a pen of 100 pigs and a 10% prevalence (10 "target" pigs), one or two pigs may be occupied elsewhere, but the remainder will contribute to the oral fluid sample. In this study, 80% of the "target pigs" in a pen interacted with the rope over pen sizes ranging from 21 to 98 pigs.

2. The sampling question ("how many ropes?") remains valid for larger pens (> 100 pigs) and more complex pen designs. All of the pens in the current study were quadrangular in shape and simple in design. That is, there were no internal barriers or barricades that affected pig movement. Therefore, the FM-inoculated pigs moved freely within the pen. Whether the same is true in pens holding large numbers of pigs or in pens designed with cul-de-sacs is not known, but could be easily determined using the methods described in this report.

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