RESEARCH REPORT
SWINE HEALTH

Title: Development of cost-efficient herd testing protocols based on testing of pooled samples using ELISA – **NPB #05-163**

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Abstract

The objective of this study was to evaluate the feasibility of pooling serum samples for detection of PRRSV by ELISA. In order to achieve this objective, 113 true positive samples and 100 false positive samples were tested undiluted and diluted 1:2, 1:4, 1:6, 1:8 and 1:10 in negative sera to estimate the Pool Sensitivity and Pool Specificity. The results were interpreted at three different ELISA cut-off values. The results show that pooling serum samples results in a decrease in Se and an increase in Sp, compared to testing individual samples, while the reduction of the s/p cut-off value recommended by the manufacturer had the opposite effect. Furthermore, we identified several combinations of pool size, cut-off value and sample size that are superior to the traditional protocol (individual samples, cut-off of 0.4) in terms of Herd Sensitivity and Herd Specificity. Therefore, the conventional monitoring protocols based on ELISA on individual samples can be improved by using pooling.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a devastating disease of swine that has an important economic impact in the swine industry in North America. Although PRRS virus (PRRSV) eradication from a single farm is feasible, it becomes very difficult, especially in high dense areas, to keep farms negative. Despite recent advances in biosecurity, PRRS outbreaks in negative farms are still common and very often the source of the introduction cannot be identified. For this reason, the concept of area eradication has been proposed in order to control PRRS, in contrast to the individual efforts of eradicating PRRSV from single farms. In the U.S., two big initiatives to control PRRS were established in 2003: the National Pork Board PRRS Initiative and the North-Central 229 Multi-State PRRS Integrated Project funded by NRI. Regional elimination is one of the objectives of both initiatives, and is the ultimate aim of the NRI funded initiative. Furthermore, the American association of swine veterinarians recently released a position statement supporting PRRSV national eradication as a long term goal.

This proposal targets the research objective number 3 of the NPB PRRSV Initiative: national PRRSV elimination. One of the main and necessary components of a regional eradication program is to monitor the status of farms where the virus has been eradicated in order to identify farms that get re-infected until the process is completed. As regional eradication programs advance, the importance of monitoring negative farms will increase, to de point of becoming the main expense. While monitoring of boar studs is usually done by PCR, monitoring of negative sow farms is performed by testing for the presence of antibodies against PRRSV in serum with a commercial indirect ELISA (IDEXX HerdChek PRRS 2XR). A recommended strategy for breeding herds is to test a representative sample on a monthly basis to achieve a probability of detection of 95%. However, the low specificity and high cost of this protocol make it inappropriate for a large scale project. Therefore, a more accurate and cost-effective monitoring system is needed in this situation.

Pooling samples has proved an effective strategy to increase the power and reduce the cost of monitoring for populations with low prevalence. Examples of the successful use of pooling for serologic tests are monitoring of Human Immunodeficiency Virus in human populations and Avian Pneumovirus in turkey populations. At the optimal pool size, pooling samples could be able to increase the sensitivity and specificity of PRRSV monitoring at the same cost, by increasing the number of sows tested.

Objectives

To determine if testing of pooled samples using ELISA may be a useful tool to decrease costs while keeping sensitivity and specificity. This objective seeks to investigate the impact of dilution of samples on the probability of false negative and false positive results.
To identify the combination of pool size, sample size, sample frequency and cut-off value that optimizes sensitivity, specificity and cost of monitoring and screening of herds for PRRS using pooling and ELISA. This objective seeks to investigate the development of cost-efficient herd testing protocols based on testing of pooled samples using ELISA.

Materials & Methods

In order to achieve these objectives, the effect of pooling on sensitivity and specificity of the ELISA test was evaluated by testing true positive and false positive samples respectively, diluted in negative sera.

Estimation of Pool Sensitivity (PSe):

For the purposes of this study we will define PSe as the probability of a pool testing positive, given one of the samples contained in the pool is positive. A total of 113 sera from 29 animals belonging to three different populations of swine experimentally inoculated with PRRSV were used to estimate PSe:

Population 1 consisted of 8 sows inoculated intramuscularly with 10^1 (4 sows) or 10^2 (4 sows) $TCID_{50}$ of PRRSV isolate MN 30-100 (Cano et al. 2007a). Serum samples taken at 14 and 24 days post-inoculation (DPI) were available from all the sows and sera obtained at 28 DPI from five sows.
Population 2 consisted of 10 6-month-old gilts and barrows inoculated intranasally with $10^{4.3}$ $TCID_{50}$ of PRRSV isolate MN-184 (Cano et al. 2007b). Serum samples were collected at 10, 14, 18 and 24 DPI. One animal died at 12 DPI and therefore only the first sample was available for this animal.
Population 3 consisted of 11 6-month-old gilts inoculated intramuscularly 10^3 $TCID_{50}$ of PRRSV isolate MN8700. Serum samples from these gilts were available at 10, 14, 18, 24 and 28 DPI.

All samples were tested undiluted and diluted 1:2, 1:4, 1:6, 1:8 and 1:10 in negative sera (sample/positive (s/p) value between 0 and 0.1) to estimate the effect of pooling in pool sizes from 2 to 10. The ELISA was performed as described by the manufacturer (IDEXX HerdChek PRRS 2XR, IDEXX Laboratories Inc, Westbrook, MA). Test results were initially interpreted as described by the manufacturer, that is, they were considered positive when s/p value was 0.4 or higher. Furthermore, the use of cut-off values of 0.3 and 0.2 was evaluated. PSe

estimates were obtained for each combination of pool size (6 pool sizes) and cut-off value (3 cut-of values). Therefore, a total of 18 PSe estimates were calculated as

$$PSe_{c,k} = NP_{c,k} / NS$$

where $NP_{c,k}$ was the number of positive samples at a given cut-off value (c) and pool size (k), and NS the total number of samples tested, which was 113.

Estimation of Pool Specificity (PSp):

A database of 13,568 ELISA results obtained with the mentioned diagnostic kit was compiled from samples sent to the University of Minnesota Veterinary Diagnostic Laboratory (UMVDL) from PRRSV-negative farms. Out of these 13,568 samples, 225, 345 and 653 false positive results were obtained at cut-off values of 0.4, 0.3 and 0.2, respectively. Therefore, the specificity of the test for undiluted samples (Sp) was determined to be 0.983, 0.975 and 0.952 for cut-off values of 0.4, 0.3 and 0.2, respectively.

In order to assess the effect of pooling on known false positive samples, 100 false positive samples were obtained from the UMVDL. These false positive samples were tested by ELISA undiluted and diluted 1:2, 1:4, 1:6, 1:8 and 1:10 in negative sera as described for the true positive samples. This would simulate the testing of pools containing one false positive sample. A PSp estimate was calculated for each cut-off value and pool size as

$$PSp_{c,k} = Sp_c + [(1-Sp_c) * (NP_{c,k} / 100)]$$

where Sp_c was the specificity of the test for undiluted samples at a given cut-off value (c); and $NP_{c,k}$ was the number of positive samples out of the 100 false positive samples tested for a given cut-off value (c) and pool size (k). It was assumed that samples with s/p values lower than 0.4 would be negative at the cut-off levels of 0.3 and 0.2 when pooled (based on data not shown from 9 samples).

Estimation of Herd Sensitivity (HSe) and Herd Specificity (HSp):

In order to investigate the development of cost-efficient herd testing protocols (objective 2), HSe and HSp were calculated for each combination of cut-off value and pool size as follows:

$$HSe_{c,k} = 1- [((1 - Prev) * PSp_{c,k}) + (Prev * (1 – PSe_{c,k}))]^n$$
$$HSp_{c,k} = (PSp_{c,k})^n$$

where n was the sample size and Prev was the proportion of animals at 10 to 28 days post-infection, which was set to 0.1.

Two different scenarios were investigated:
Scenario 1: twenty-four samples were collected from a herd presumably free of PRRSV. The samples were run either individually (24 ELISA tests), in pools of 2 (12 tests), in pools of 4 (6 tests), in pools of 6 (4 tests) or in pools of 8 (3 tests). Under this scenario the number of samples was constant and the number of tests depended on the pool size.
Scenario 2: ten to 100 samples were collected from a herd presumably free of PRRSV. A total of 10 ELISA tests were run on individual samples (n=10), or in pools of 2 to 10 (n=20 to 100 respectively). Under this scenario the number of tests was constant (10 tests) and the number of samples tested depended on the pool size.

Results

Estimation of PSe and PSp (objective 1):

Estimation of PSe: ten samples, out of the 113 true positive samples, tested negative. Among the rest of the samples, s/p values ranged from 0.447 to 2.986 (Fig 1).

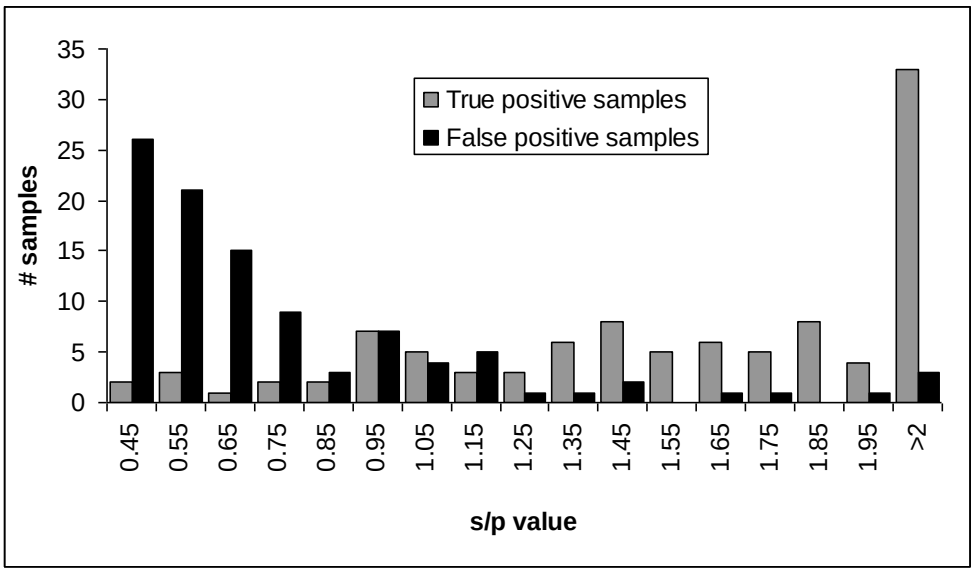

Figure 1: Distribution of the ELISA s/p values.

Population 1 showed the highest s/p values at 14 DPI with an average of 2.066 and then decreased (Fig 2). A different pattern was observed for populations 2 and 3, with average s/p values increasing up to around 1.7 at 18 to 24 DPI. It is important to note that a large variability was observed among animals from the same population. For example, in population 3 for one animal the highest s/p value observed was 0.575, while for another animal the lowest s/p value was 1.756.

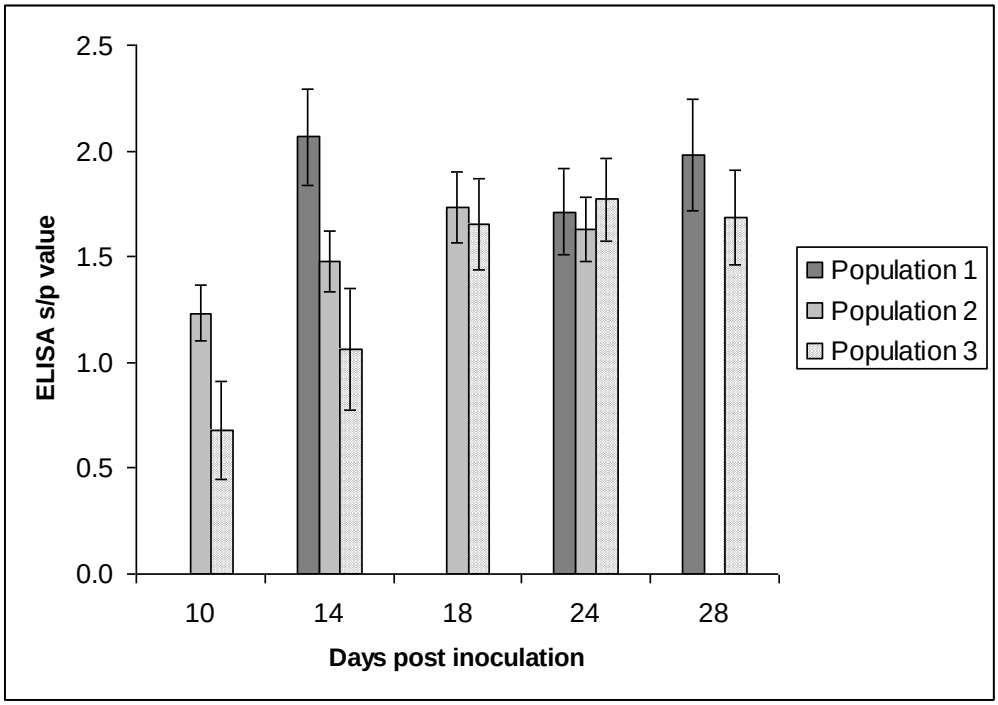

Figure 2: Comparison of the average ELISA s/p value obtained from sera from three different populations of swine experimentally infected with PRRSV, at different times post inoculation.

When samples from all the populations and sampling times were considered as a whole, PSe estimates ranged from 0.92 (undiluted sample, cut-off 0.2) to 0.42 (pools of 10, cut-off 0.4) (Table 1). Figure3 shows the effect of pool size and cut-off value on PSe. For all three cut-off values, an increase in the pool size was associated with a decrease in the sensitivity. On the other hand, lower cut-off values resulted in higher sensitivities.

Table 1: Pool Sensitivity estimates (in %).

	Cut-off		
Pool size	**0.4**	**0.3**	**0.2**
1	91	92	92
2	84	88	90
4	69	79	84
6	58	70	79
8	48	61	71
10	42	51	65

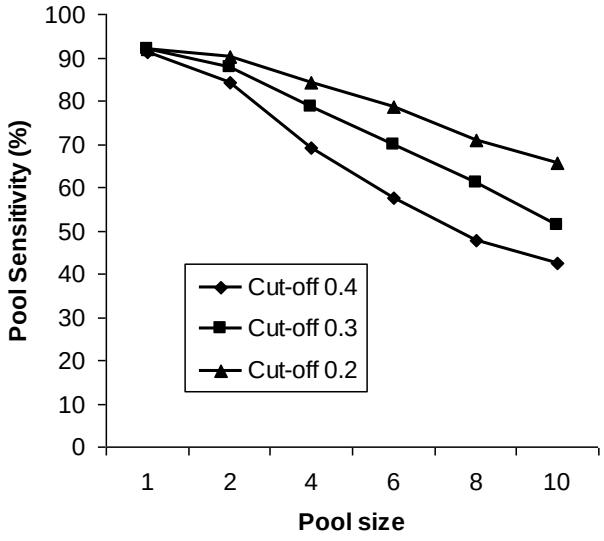

Figure 3: Effect of the pool size and the cut-off value on the Pool Sensitivity.

Estimation of PSp: the distribution of the s/p values of the 100 false positive samples is shown in figure 1. The PSp estimates ranged from 0.952 (individual, cut-off 0.2) to 0.999 (pools of 10, cut-off 0.4) (Table 2). The effects of the pool size and the cut-off value on PSp are shown in figure 4. PSp increased with pool size. Moreover, PSp estimates were highest for a cut-off of 0.4 and lowest for a cut-off of 0.2.

Table 2: Pool Specificity estimates (in %).

	Cut-off		
Pool size	**0.4**	**0.3**	**0.2**
1	98.34	97.46	95.19
2	99.40	99.07	98.46
4	99.85	99.77	99.49
6	99.88	99.85	99.68
8	99.93	99.85	99.82
10	99.95	99.88	99.83

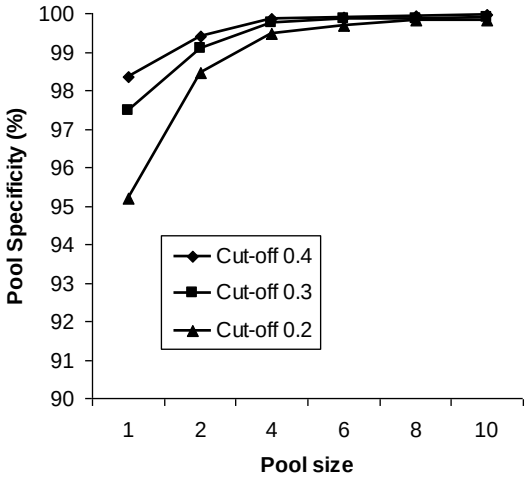

Figure 4: Effect of the pool size and the cut-off value on the Pool Specificity.

Estimation of HSe and HSp (objective 2):

In the first scenario, HSe and HSp were calculated for different sampling protocols using 24 samples. The estimates of HSe and HSp for different pool sizes and cut-off values are shown in figure 5. For the protocol using the current standards (undiluted samples and a cut-off of 0.4) HSe and HSp were estimated as 0.94 and 0.61, respectively. When samples were run pooled, HSe decreased and HSp increased. Alternatively, when a cut-off value lower than the standard was used, HSe increased and HSp decreased.

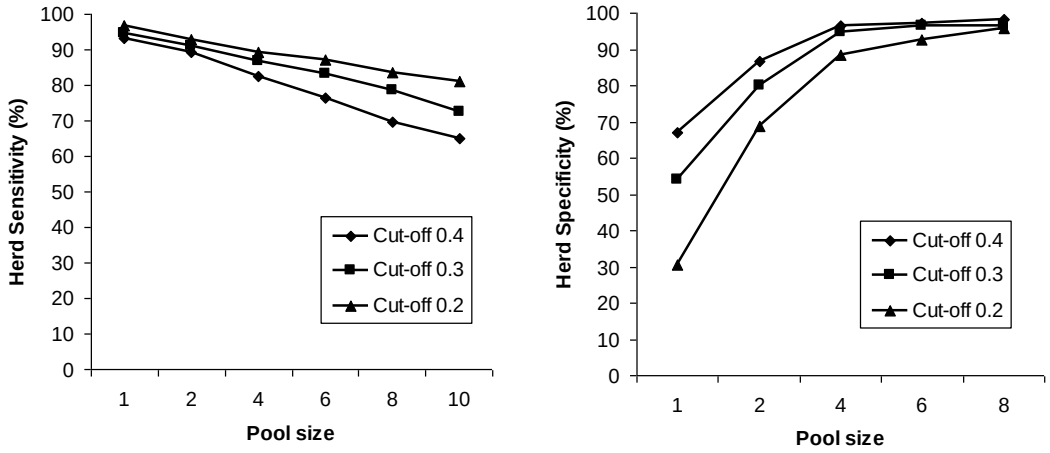

Figure 5: Effect of the pool size and the cut-off value on Herd Sensitivity and Herd Specificity under the first scenario.

In the second scenario, the number of animals sampled was determined based on the pool size and a fixed number of 10 ELISA tests. Figure 6 show the HSe and HSp estimates for the different combinations of pool size and cut-off value. The protocol testing 10 individual samples and using the cut-off value of 0.4 showed a HSe of 0.62

and a HSp of 0.85. HSe increased with the pool size due to the larger number of animals sampled. HSp increased with the pool size up to a pool size of 4 and remained almost constant at higher pool sizes. When the cut-off value was reduced, a decrease in the HSp and an increase in the HSe were observed. Most of the protocols evaluated were superior to the standard protocol in terms of HSe and HSp.

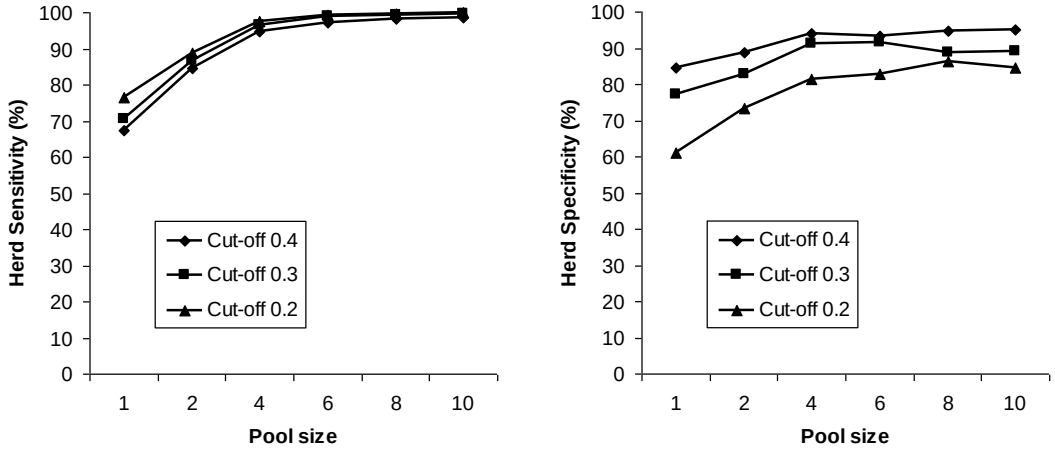

Figure 6: Effect of the pool size and the cut-off value on Herd Sensitivity and Herd Specificity under the second scenario.

Discussion

In this study, the feasibility of using pooled samples to detect PRRSV with a commercial ELISA at different cut-off values was evaluated. The results show that pooling serum samples results in a decrease in Se and an increase in Sp, compared to testing individual samples, while the reduction of the s/p cut-off value recommended by the manufacturer had the opposite effect. An important finding of the present study is that PSp increases with the pool size because of a dilution effect on false positive samples. Although this phenomenon has been reported before for other serological tests, it is often overlooked in studies evaluating the performance of diagnostic tests on pooled samples, which are mainly concerned about the effect on sensitivity. Current surveillance protocols for breeding herds that are negative to PRRSV use ELISA on individual serum samples. For such a protocol to have a high HSe it is necessary to sample a large number of animals. Unfortunately, the drawback of that strategy is a decrease in the HSp. In fact, false positive results are common in sets of samples from negative herds and these samples have to be confirmed negative by other tests such as IFA or PCR. Another strategy to increase the HSe of the protocol would be to decrease the cut-off value of the test. Previous studies investigated the effect of the cut-off value on the sensitivity and specificity of the ELISA. However, as reported in those studies and in the present work, a decrease in the cut-off would also increase the number of false positives.

In the present study we describe a way to increase the HSe of a surveillance protocol for breeding herds and at the same time increase its HSp, without increasing the testing costs. This can be achieved by sampling a larger number of animals and running the samples in pools, as shown in the second scenario evaluated. In that example, testing samples in pools of sizes from 2 to 10 resulted in protocols with higher HSe and HSp than testing samples individually. Since the number of ELISA tests was kept constant (10 tests), all the evaluated protocols had the same testing costs. On the other hand, because the protocols using pooling require sampling more animals, these protocols would have higher sampling costs (labor and materials). It is important to note that HSe and HSp calculated under scenario 1 and scenario 2 are just examples of the application of the obtained estimates of PSe and PSp. Nevertheless, similar scenarios were evaluated with different sample sizes (from 1 to 100), number of ELISA tests (form 1 to 50) and prevalences (from 0.01 to 0.5). Under all these scenarios, although different HSe and HSp estimates were obtained (data not shown), the conclusions remained the same as for the illustrated examples. Furthermore, using the PSe and PSp estimates and the formulas provided in this manuscript, the reader should be able to find HSe and HSp for any particular scenario of interest.

Whether PRRSV regional eradication projects are going to be implemented, an accurate and cost-effective sampling protocol is going to be needed to monitor the PRRSV status of negative farms. Results reported in this study show that the conventional monitoring protocols based on ELISA on individual samples can be improved by using pooling.

Lay Interpretation

The objective of this study was to evaluate the feasibility of using pooled serum samples for detection of PRRSV-infected sow herds by ELISA. In order to achieve this objective, 113 true positive samples and 100 false positive samples were tested undiluted and diluted 1:2, 1:4, 1:6, 1:8 and 1:10 in negative sera to estimate the effect of pooling on the accuracy of the ELISA test. Results show that pooling of a single truly positive sample with negative samples may result in an ELISA negative test and false negative result. However, this dilution effect can also decrease the likelihood false positive results, compared to testing individual samples. Furthermore, we found that by pooling samples and increasing the number of animals sampled, we can increase the accuracy of the monitoring protocol at the same testing costs. Therefore, the conventional monitoring protocols based on ELISA on individual samples can be improved by using pooling.