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Comparison of Pooled Testing to Individual Testing with an Ear-notch Antigen-capture Enzyme-linked Immunosorbent Assay for Bovine Viral Diarrhea Virus

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Abstract

Thirty-seven skin samples (ear notches) from individual antigen-capture enzyme-linked immunosorbent assay (ACE)-positive cattle were collected and pooled with negative samples in pools of two, three, four, five, 10 and 15 samples to experimentally determine an optimum pool size for the testing protocol for bovine viral diarrhea virus (BVDV). All positive BVDV pooled samples containing ACE-positive skin specimens resulted in positive tests for all pool sizes investigated. Samples in the 15 ear-notch pools, despite remaining positive, approached the detection limit of the assay. An economic analysis of pooled ACE testing was undertaken to determine the most economically rewarding pool size within the constraints of the test. The analysis demonstrated that as BVD PI prevalence increased, optimal pool size decreased. In all cases, a point of diminishing returns was seen until there was a reversal of the trend, and an increased pool size increased the cost of testing on a per-head basis regardless of BVD PI prevalence. Given the results from the economic analysis and the evaluation of ACE pooled testing, these data suggest that pool sizes of five to 10 samples may be most advantageous both economically and for reliably to detect persistently infected animals.

Keywords: bovine, BVDV, persistent infection, ear-notch

Résumé

Un total de 37 échantillons cutanés d'oreilles, provenant de bovins testant positifs suite à un essai immunoabsorbant lié à l'enzyme (ELISA) pour la détection des antigènes, ont été ramassés et regroupés avec des échantillons négatifs dans des ensembles de deux, trois, quatre, cinq, 10 et 15 échantillons pour déterminer expérimentalement la taille optimale des regroupements dans les protocoles servant à détecter le virus de la diarrhée virale bovine (BVDV). Tous les échantillons regroupés positifs au BVDV qui contenaient des échantillons positifs suite à l'ELISA ont donné des tests positifs pour toutes les tailles de regroupement examinées. Les échantillons dans les regroupements de 15 morceaux d'oreilles bien que testant positifs approchaient la limite de détection de l'essai. Une analyse économique de la méthode de regroupement basée sur l'ELISA a été faite pour déterminer la taille des regroupements la plus viable économiquement prenant en ligne de compte les contraintes du test. L'analyse a démontré que lorsque la prévalence d'animaux immunotolérants au BVD augmentait, la taille optimale des regroupements diminuait. Dans tous les cas, le rendement décroissait avant que la tendance ne change de direction de sorte que l'accroissement de la taille des regroupements augmentait le coût du test par unité animale peu importe la prévalence des animaux immunotolérants au BVD. À la lumière des résultats de l'analyse économique et de l'évaluation de la méthode de regroupement basée sur l'ELISA, il semble que les tailles de regroupement comportant de cinq à 10 échantillons soient les plus avantageuses autant du point de vue économique que du point de vue de l'aptitude à détecter les animaux immunotolérants.

Introduction

Bovine viral diarrhea virus (BVDV) is a Pestivirus in cattle which causes negative economic impacts on the beef industry.[12] This virus can affect all stages of production, impacting reproduction, calfhood health and

feedlot performance.[5,6] The identification and removal of animals persistently infected (PI) with BVDV from a production system is a key component to controlling BVDV.[1,4,10] In order to achieve this, a reliable method of testing for PI animals must be used.[3] Ear notching to obtain skin samples is a common and easy way to obtain diagnostic samples in a relatively non-invasive manner. These tissue samples can be examined by immunohistochemistry (IHC) for presence of the BVD virus in the epithelium. Recent advancements allow the testing of a phosphate buffered saline (PBS) ear-notch extract by antigen-capture enzyme-linked immunosorbent assay (ACE).[9,11] This test for detecting BVD-infected animals is much more rapid than the conventional IHC testing. Additionally, ELISA testing of ear-notch samples does not require specialized equipment or personnel that IHC or polymerase chain reaction require, and is readily adapted to automation. Despite the ease of ACE testing to identify PI individuals, it can be cost prohibitive for whole-herd screening. To address this, use of pooled testing of samples by ACE is being explored to make herd-wide testing more economical. In order to realize the benefits of pooling, the level at which pooled testing is effective must be established. The economic benefits of pooling will not be appreciated if the pooling method compromises the sensitivity of the test. The objective of this study was to determine the effect of pooled sampling on sensitivity of ACE testing, and to assess the potential economic benefits of the pool sizes utilized.

Materials and Methods

Skin samples (ear notches) were collected from 37 BVD PI calves and three calves that were not persistently infected with BVD. Ear notches for this study were collected from research animals from another study at Kansas State University, New Mexico State University and field samples collected by Great Bend Animal Medical Center in Great Bend, KS. All samples were chilled and stored in 2 mL of PBS. The BVD PI status of all calves had been previously determined by individual ACE testing, and all samples were retested using ACE upon arrival at the Kansas State University Veterinary Diagnostic Laboratory (KSUVDL). Additionally, approximately 5,500 PI-negative ear notches were collected from feedlot populations (Dr. Shaun Sweiger, Edmond, OK) under field sampling conditions and used to configure sample pools. These samples were confirmed BVD PI-negative by at least two individual ACE tests, the final being conducted at the KSUVDL.

Pools were configured to contain an aliquot from each of the 40 experimental samples and pooled with the BVD PI-negative samples to form pools of 2, 3, 4, 5, 10 and 15 samples. One-hundred microliters of supernatant from each of the 37 positive and three negative ear-notch samples was placed in a separate tube. Then equal aliquots (100 µl) of supernatant from random known negative ear-notch samples were placed in the pooling tube with the test samples until the desired number of pooled samples was achieved. None of the 5,500 known negative ear-notch samples used for pooling purposes were utilized twice in the study. Pooled sample tubes were vortexed and pooled samples were later transferred to the test wells for the ACE test. Aside from the experimental samples, no individual sample was used in a pool more than once in order to closely mimic the variation among the negative population in a field-testing situation.

Antigen-Capture ELISA test

The ACE testing procedures were followed per test kit instructions supplied by the manufacturer.[a] All microwells were prewashed with approximately 0.2 mL of ELISA wash buffer, which was then decanted and discarded. PBS extract (0.1 mL) was pipetted into a microwell for pooling. The wells were then covered with an adhesive film and allowed to incubate at room temperature for 1.5 hours. After incubation, the PBS extract was removed from the wells, and wells were washed three times by adding 0.2 mL of ELISA wash buffer, discarding the buffer with each wash. Following this, 0.1 mL of working detector reagent was added to each well. Wells were then re-covered and incubated at room temperature for 1.5 hours. The working detector reagent was discarded after incubation, and three washes were performed with 0.2 mL ELISA wash buffer as before. After the wash was completed, 0.1 mL of Enzyme Conjugate Reagent (ECR) was added to each well; wells were covered and incubated at room temperature for one hour. During incubation of the enzyme conjugate, the 3,3',5,5' – tetramethylbenzidine (TMB) substrate reagent and stop solution were allowed to equilibrate to room temperature for approximately one hour. At the conclusion of the enzyme conjugate incubation period, the ECR was removed from the wells and wells were washed three times with buffer solution. Then 0.1 mL of the TMB substrate reagent was added to each well; wells were covered and incubated for 10 minutes in a dark area. Stop solution (0.1 mL) was added to each microwell at the conclusion of the incubation period. Wells were then re-covered and incubated for 10 minutes in a dark area. Following the stop solution, incubation spectrophotometric reading of the microwells was performed at an absorbance of 450 nm, utilizing water blank. Normalized optical densities (OD) were determined by the following calculation:

$$\text{Normalized OD} = \frac{\text{raw OD of sample} - \text{raw OD of negative control}}{\text{raw OD of positive control} - \text{raw OD of negative control}}$$

Positive and negative sample results were determined by comparing these normalized ODs (referred to as S/P ratios) to standard cutoffs provided by the test manufacturer[a] as follows: normalized ODs less than 0.20 indicate BVDV-negative samples; normalized ODs in the range of 0.20 to 0.39 are considered "gray zone" samples that must be retested; and normalized ODs greater than 0.39 indicate samples that are BVDV-positive.

Data Analysis

Data were analyzed in SAS® version 9.1 (SAS Institute Inc., Cary, NC) and $P \leq 0.05$ was used for all hypothesis testing. We used generalized linear mixed models in Proc MIXED to compare mean S/P ratios using a repeated measures option to account for the effect of multiple samples per animal. Model generated least-square means were used for all two-way comparisons.

Economic Analysis

The economic effect of pooling was evaluated utilizing the formula:

$$E(C_{herd}) = rc[(k+1) - k(1-\pi)^k]$$

where $E(C_{herd})$ is the total cost of testing a herd; r is the number of pools; c is the cost of the test; k is the number of individuals in the pool; and π is the expected prevalence of BVD for that herd. This formula was adapted from work done by Munoz-Zanzi *et al*[7] on the economics of pooled PCR BVD testing. The formula includes factors for adjustment in cost based on prevalence, pool size, initial testing cost and cost of re-tested pools for confirmation of positive individuals. It does not account for differences in sensitivity or specificity of a test.

This formula was inputted into a Microsoft Excel® spreadsheet to allow for manipulation of the variables affecting total cost of herd-wide testing. Herd size of 1000 head was used for this analysis to determine per-head cost, and number of pools based on desired pool size. Although the formula results in total herd cost, results were reported on a per-head basis for comparison. Analyses were run for cost per head for variations in prevalence and pool size.

Results

Pool size affected mean S/P ratios of the ACE test (Figure 1; $P<0.01$). Most notably, mean S/P ratios for both the 1:10 (all P-values <0.01) and 1:15 (all P-values <0.01) pool sizes were significantly lower than means for all smaller pool sizes. Although differences occurred, at no time did a positive pool result in a suspect or negative test (Figure 2). Individual samples varied in their S/P ratios at different dilutions. Some approached the lower limit of detectible positive in the 1:15 pool size (Figure 1).

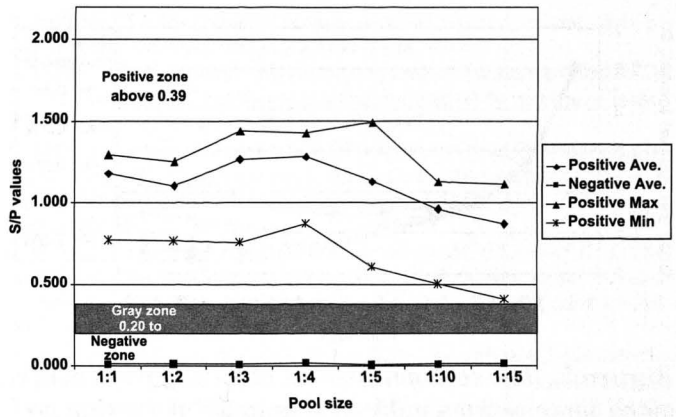

Figure 1. Maximum, minimum and mean observed S/P ratio for pools of different sizes as compared to the test cutoff for positive results with the BVD antigen-capture ELISA testing method.

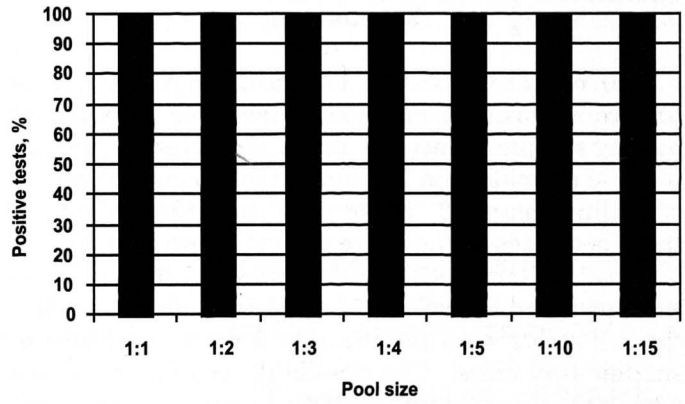

Figure 2. Percentage of known BVD PI-positive ear-notch samples that tested positive when pooled with known BVD PI-negative samples utilizing the antigen-capture ELISA test.

The two lowest values for S/P ratios were observed when positive samples were tested in pool sizes of 10 and 15 individuals (Figure 1). However, the means remained strongly in the positive zone across all pool sizes.

When the economic analysis was conducted for several prevalence values that have been shown to be present in different sectors of the US beef industry,[6] a clear point of reversal in economic benefit was present for each prevalence level (Figure 3). As prevalence increased, the benefit of larger pools decreased economically. Also, as the number of samples pooled increased, the economic benefit decreased until there was a reversal of trend, resulting in increased cost with increased numbers of samples in the pool.

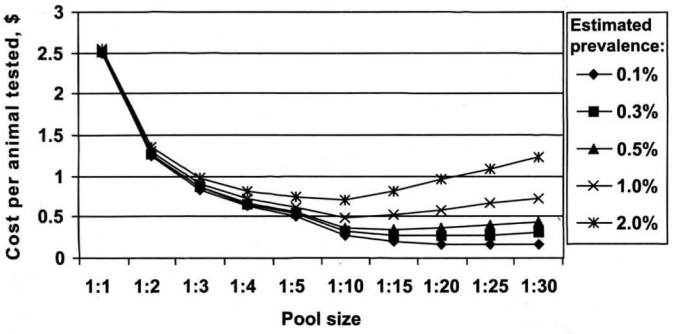

Figure 3. The economic benefit of testing pools of ear-notch samples from individual animals at varying pool sizes based on the expected prevalence (0.1, 0.3, 0.5, 1.0 and 2.0%) of BVD PI animals in the herd. Costs are shown on a dollar-per-head basis and include the cost of both screening and confirmatory testing for a given herd, utilizing a diagnostic tool that costs $2.50 per test.

Discussion

In order to be confident in the use of pooled testing protocols, two questions must be answered: 1) how does pooling samples affect the ability of the test to identify positive animals, and 2) what is the economical return of pooling samples? These data show that pooling at these pool sizes did not have a negative effect on detecting positive samples. However, when positive samples were included in pools of 10 or 15 individual samples, the S/P ratios were significantly decreased relative to smaller pool sizes. The possibility of false negatives appears to become more realistic as pool size increases. This and several other studies have shown that as the pool size increases, the chance of increased false-negative test results becomes a very real possibility. In a study conducted in 2006 by Cleveland *et al*,[2] there were significant differences in sensitivity of tests when they were converted from an individual to a pooled testing protocol. In Munoz-Zanzi *et al*,[7] researchers reported a trend towards decreased sensitivity as pool sizes increased in pooled PCR testing of blood samples.

In many instances the benefit of large pools may not outweigh the risk. Some elements to consider when selecting pool size include the expected prevalence of BVD PI cattle in the individual operation, the size of the pool which can be used and the overall cost of false-positive or negative individuals. In feedlot settings, where prevalence has been shown to approximate 0.3%,[6] the point of greatest economic return to pooling is at 20 head per pool; however, it is important to note that reducing the pool size to 15 samples only raises the test cost by one cent per head, and further reducing the pool size to 10 increases the cost by five cents per head. Utilizing a pool size of five, the safer alternative in terms of sensitivity, increases the cost to $0.56 per head, compared to $0.27 at the least-cost pool size of 20. Loneragan *et al*[6] showed that increased morbidity attributable to a PI animal in the population increased by 43% over the feeding period. This would result in increased cost of treatment, chronically ill animals and mortality, and may indicate it may be more economical to invest in testing in order to be confident that all BVD PI animals have been identified and removed from the population.

Munoz-Zanzi *et al*[7] reported the effects of different pooling methods with different diagnostic tests to identify BVD PI cattle. Their model included prevalence estimates for BVD PI cattle ranging from 1 to 10%. Our study was conducted using similar modeling, but included BVD PI prevalence estimates that would more closely mimic a feedlot setting.[6,8] Accurate estimates of herd prevalence are important when designing pooled testing protocols. If the prevalence estimate is not accurate, the benefit of pooling may not be as readily evident. An increase in prevalence from 0.3% to only 0.5% (two additional PI individuals in a 1000-head herd) changes the pooling size with the greatest economic benefit from 20 head to 15 head. A further increase to 1% prevalence (10 total PI individuals in a 1000-head herd) moves the point of greatest return to a pool size of 10 individuals.

The economic benefit of large pools increases as the prevalence of BVD PI decreases. When prevalence is low it becomes important to balance the benefit of pooling with the increased chance of obtaining false negative results. These data show that the populations with the lowest prevalence benefit most from large-pool testing, whereas sectors or even individual operations with suspected high prevalence may need to consider smaller pool sizes when testing in order to maximize the economic returns on pooled testing. Therefore, in situations where the prevalence is in question it appears to be most advantageous to use a smaller pool size of five to 10 head, where both the sensitivity of the test as well as the potential economic return can be optimized.

Conclusions

These data support pooling of samples when utilizing ACE testing to identify BVD PI cattle. The S/P ratios of pooled samples were significantly lower when more than five samples were pooled together. However, none of the pool sizes—up to 15 samples—resulted in positive samples being diagnosed as a false negative. These data also show that as BVD PI prevalence increases, the number of samples included in the pool should be reduced due to cost of testing the herd. The recommendation from this study is that pooling of up to five to 10 samples will yield the greatest economic impact, decreasing the

cost of testing a group of cattle, without decreasing the ability of the test to identify positive individuals.

Endnote

[a]IDEXX HerdChek* BVDV Antigen/Serum Plus Test Kit, IDEXX Laboratories, Inc., Westbrook, ME

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