PEER REVIEWED

Effects of Ear-Notch Sample Handling on Reliability of Antigen-Capture Enzyme-Linked Immunosorbent Assay Testing for Bovine Viral Diarrhea Virus

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Abstract

Antigen-capture enzyme-linked immunosorbent assay (ACE) testing of skin samples obtained by ear-notching is commonly used to detect animals persistently infected (PI) with bovine viral diarrhea virus (BVDV). At times practitioners may not have access to phosphate buffered saline (PBS) solution for specimen storage as suggested by the ACE kit manufacturer. The first objective of this study was to observe the effects of storing ear-notch samples in varying amounts of PBS on ACE results. The second objective was to observe the effects of delayed time before the ear-notch sample was placed in PBS on the ACE results. Using virus isolation and immunohistochemistry, nine PI-positive and eight PI-negative calves were identified to obtain ear notch biopsies for the study. The amount of PBS used to store both positive and negative ear-notch samples was varied (1, 2, 4, 6, 8 and 10 mL) in the first trial. In the second trial, the addition of PBS to both positive and negative samples was delayed post-collection; phosphate buffered saline was added to ear-notch samples at 0, 1, 2, 4, 8, 12 and 24 hours post-collection. Following sampling and application of treatments, all samples were tested in duplicate for BVDV antigen following the guidelines of the ACE kit manufacturer. Although the S/P ratios were different among treatment groups in both studies ($P<0.05$), neither varying the amount of PBS added to the sample vial nor delaying the addition of PBS to the sample affected the outcome when testing positive or negative samples.

Résumé

Un essai immunoabsorbant lié à l'enzyme (ELISA) pour la détection des antigènes à partir d'échantillons cutanés d'oreilles est utilisé couramment pour identifier les animaux immunotolérants (PI) au virus de la diarrhée virale bovine (BVD). Il arrive parfois que les praticiens n'aient pas accès à une solution saline phosphate tampon (PBS) pour l'entreposage des échantillons tel que suggéré par le fabricant du test. Le premier objectif de nos études était de déterminer l'effet d'entreposer les échantillons cutanés d'oreilles dans différentes quantités de solution saline phosphate tampon sur les résultats du test ELISA. Le second objectif était de déterminer l'effet d'un délai dans l'entreposage des échantillons cutanés d'oreilles dans la solution tampon sur les résultats du test ELISA. À l'aide de l'isolation virale et de l'immunohistochimie, on a identifié neuf individus immunotolérants et huit individus négatifs desquels ont été prélevés des échantillons cutanés d'oreilles pour cette étude. La quantité de PBS utilisée pour entreposer les échantillons cutanés d'oreilles positifs et négatifs a été modifiée (1, 2, 4, 6, 8 et 10 ml) dans la première étude. Dans la seconde étude, l'addition de PBS aux échantillons positifs et négatifs a été retardée après l'échantillonnage par 0, 1, 2, 4, 8, 12 ou 24 heures. Après l'échantillonnage et l'application des traitements, tous les échantillons ont été testés en duplicata pour détecter la présence d'antigène du virus de la diarrhée virale bovine selon les directives du fabricant du test (ACB). Bien que le rapport S/P variait entre les traitements dans chacune des études ($P<0.05$), la capacité du test ELISA a identifié les échantillons positifs ou négatifs n'était influencée ni par la variation dans la quantité de la solution saline tampon utilisée ni par le délai dans l'addition de la solution tampon.

Introduction

Bovine viral diarrhea virus (BVDV) has a negative health impact on cattle throughout all segments of the beef industry, resulting in decreased profits for cattle

producers.[7,8,13] If the fetus is infected with BVDV prior to immune system development, the calf will be born persistently infected (PI) with BVDV.[7] The beef industry has become more aware of controlling BVDV through the identification and removal of persistently infected (PI) animals from a production system.[10]

Skin or ear-notch samples from cattle can be utilized to determine if an animal is persistently infected with BVDV.[10,12] Tests utilized to identify BVDV antigen in skin include immunohistochemistry (IHC),[1,4,5,9] antigen-capture enzyme-linked immunosorbent assay (ACE)[3,6] and polymerase chain reaction (PCR).[2] Samples destined for ACE testing are stored in phosphate buffered saline (PBS), and the ear-notch extract (supernatant) can be utilized for the ACE test.[10,11,12]

The manufacturer of a commercially available ACE test[a] recommends that ear-notch samples be placed immediately in PBS solution and stored at 35.6 to 46.4°F (2 to 8°C), and that samples be sent to the testing laboratory within 48 hours. Practitioners encounter situations when ear-notch diagnostic samples might be collected, but appropriate equipment or sample storage containers with media are unavailable. We have received many calls from veterinarians in the field asking if samples would be suitable for diagnostic evaluation if the manufacturer's handling recommendations were not followed. Therefore, the objectives of this study were: 1) to determine the effects of varying amounts of PBS in a vial when storing bovine ear-notch samples for ACE and 2) to determine if ACE test results differ when ear-notch samples are placed in PBS solution at different times after collection.

Materials and Methods

Nine BVDV PI calves were being housed at Kansas State University for a food safety challenge study. The calves were four to five months of age and were from the same herd located in southwest Kansas. Genetic diversity of the persistent strains of BVDV were not tested, but with all calves originating from the same herd it is unlikely there was much diversity between strains infecting the calves. Negative BVDV virus samples were taken from control calves being utilized in the same study. Ears from BVDV PI positive and negative calves were collected at necropsy and transported to a laboratory located in the Department of Clinical Sciences at Kansas State University for processing.

Trial 1 was conducted to determine the effect of adding varying amounts of PBS to ear-notch samples on the sensitivity of the ACE test. Ears were collected from nine known PI animals and four known PI-negative animals at necropsy. These ears were notched to yield 1 cm by 1 cm tissue samples, and samples were placed in 0.1 M PBS. Either 1, 2, 4, 6, 8 or 10 mL of PBS was added to each of the notches collected from each animal. Samples were stored in PBS for 48 hours, after which all samples were tested for BVDV utilizing ACE.

Trial 2 was conducted to determine the effect of delaying the addition of PBS to ear-notch samples on the sensitivity of the ACE test. Ears were collected from nine known PI animals and eight known PI-negative animals at necropsy. Ears were notched in 1 cm by 1 cm samples, and tissue was placed in tubes and exposed to room air at room temperature prior to adding 0.1 M PBS at specific intervals. PBS was added to samples at 0, 1, 4, 8, 12 and 24 hours post-notching. All samples were subsequently tested for BVDV utilizing ACE.

The ACE test[a] was utilized in both Trial 1 and Trial 2. All tests were run in duplicate and according to manufacturer's recommendations. All microwells used were prewashed with approximately 0.2 mL of ELISA wash buffer, which was then decanted and discarded. One hundred microliters of serum was pipetted into a microwell for each sample. Wells were covered with an adhesive film and allowed to incubate at room temperature for one hour. After incubation, serum 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 one hour. 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, 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 tetramethylbenzidine (TMB) substrate reagent and stop solution were allowed to equilibrate to room temperature for approximately one hour. At conclusion of the enzyme conjugate incubation period, the ECR was removed from the wells, which were then washed with buffer solution. After adding 0.1 mL of TMB substrate reagent to each well, wells were covered and incubated for 10 minutes in a dark area. At the conclusion of the incubation period, 0.1 mL of stop solution was added to each microwell. Wells were then re-covered and incubated for 10 minutes in a dark area. Spectrophotometric reading of the microwells was performed at an absorbance of 450 nm, utilizing a 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 to standard

provided by IDEXX Laboratories, Inc. as follows: normalized ODs less than 0.20 indicated BVDV-negative samples; normalized ODs in the range of 0.20 to 0.39 were considered "gray zone" samples that must be retested; normalized ODs greater than 0.39 indicated BVDV-positive samples.

Samples determined to be "gray zone" were retested using a modified protocol that utilized a modified working detector reagent that contained no detector antibody. Aside from the substitution of this modified working reagent for the normal working detector reagent, ACE tests were run as described above.

Calculations of normalized ODs for "gray zone" retesting utilized the formula:

$$\text{Normalized OD} = \frac{\text{raw OD of sample with detector} - \text{raw OD of sample without detector}}{\text{raw OD of pos control, no detector} - \text{raw OD of neg control, no detector}}$$

These calculated normalized ODs were compared to the standards: ODs less than 0.20 indicated BVDV-negative samples, and those greater than 0.20 indicated BVDV-positive samples.

Statistical Analysis

All data analyses were performed using a statistical software package[b] and $P \leq 0.05$ was used for all hypothesis testing. Logistic regression in Proc GENMOD was used to model the probability of samples testing positive for different treatment groups. We compared mean S/P ratios using generalized linear mixed models in Proc MIXED. In all models, a random statement was used to account for the effect of multiple samples per animal. Model generated least-square means were used for all two-way comparisons when significant treatment effects were observed.

Results

In Trial 1, there was a BVDV status-by-mL-of-PBS-added interaction ($P<0.01$; Figure 1) for S/P ratio from the ACE test. As the amount of PBS added to a sample was increased, the mean S/P ratio for positive BVD PI ear notches decreased. However, different amounts of PBS added to sample tubes had no effect on S/P ratios for negative BVD PI ear-notch ACE test results. BVD PI-positive samples stored in 2 mL of PBS had similar S/P ratios as samples stored in 1 mL or 4 mL of the solution. BVD PI-positive samples stored in 6, 8 or 10 mL of PBS had significantly lower S/P ratios ($P<0.05$) compared to samples stored in 1, 2 or 4 mL of PBS. Despite this, as seen in Figure 2, there was no significant difference in the ability of the test to detect positive individuals across all treatments.

There were no differences in test outcomes caused by delayed PBS addition interactions in Trial 2, therefore only main effects are reported. These data indicate that delaying the addition of PBS to ear-notch samples did not affect the detection rate for BVD, which remained at 100% across all treatments. However, there were differences in the S/P ratios at different treatment levels (Figure 3). The lowest S/P ratios occurred when PBS was added to samples at 2, 4 or 8 hours after the ear-notch was collected ($P<0.05$). The highest S/P ratios occurred when PBS was added immediately, 1 hour, 12 hours, or 24 hours after collection of the samples. Although there were differences, no mean S/P ratios from the different times PBS was added approached the positive S/P ratio threshold of the ACE test kit.

Discussion

There are times in veterinary practice where diagnostic samples are mishandled. This study tried to simulate common errors encountered when practitioners

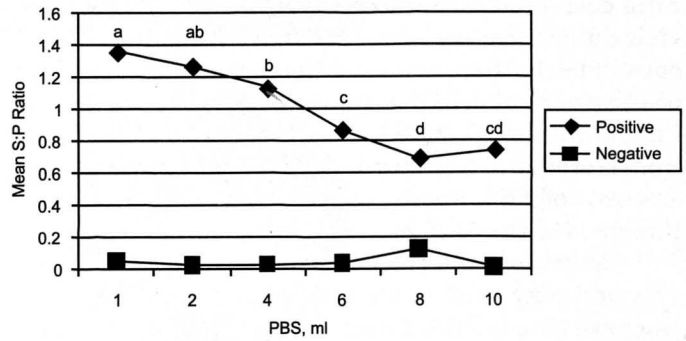

Figure 1. Effect of different amounts of PBS added to a sample tube on the S/P ratio utilizing the ACE BVD test (BVD status x mL of PBS interaction, $P<0.01$; means with a different superscript differ $P<0.05$).

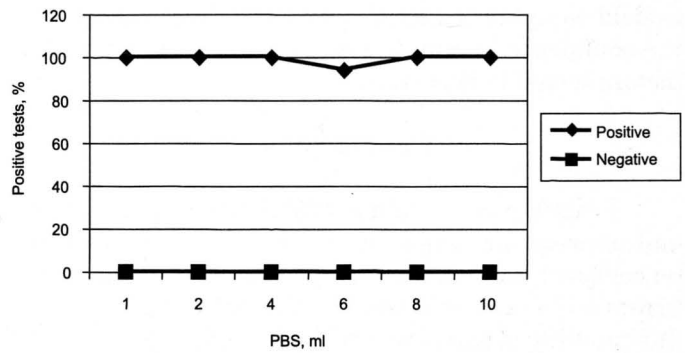

Figure 2. Effect of different amounts of PBS added to a sample tube on the percentage of tests correctly identified as positive.

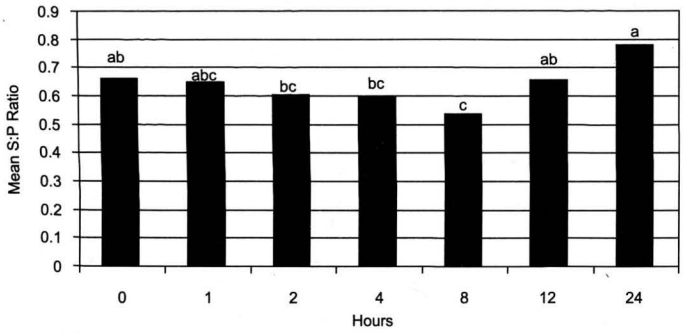

Figure 3. Effect of different time intervals until PBS was added to samples allowed to sit at room temperature post-collection on the S/P ratio utilizing the ACE BVD test. Means with different letters differ ($P<0.05$).

call with sample questions. Diluting the sample with additional PBS at the time of sampling had a significant effect on the S/P ratios of the tests. The manufacturer of the ACE test kit we utilized recommends that samples be stored in 2 mL of PBS, however there was not a significant decrease in S/P ratio until 6 mL or more of PBS was added to the ear notches. Although the S/P ratio decreased, additional amounts of PBS in sample vials did not decrease the sensitivity of the test. These data indicate there was no difference in S/P ratio from positive ear-notch ACE samples stored in 1, 2 or 4 mL of PBS, and although the S/P ratio decreased when samples were stored in 6, 8 or 10 mL of PBS relative to the lower amounts of PBS, results did not approach the positive threshold of the ACE kit.

Sometimes veterinarians may not have PBS available and may need to know how long one can wait to place samples in PBS and refrigerate. This study tried to simulate field conditions in the laboratory, ranging from no delay up to a 24-hour delay until the PBS was added to samples. Delaying the addition of PBS affected the S/P ratio, however, positive samples were still detected using ACE even when PBS was not added for up to 24 hours after sample collection. This is important to know in field sampling situations where sample storage and the confidence in results may be questioned due to the factors tested in this study.

Conclusions

Practitioners should always strive to supply fresh and appropriate diagnostic samples. Samples should be collected and handled as directed by test manufacturers and diagnosticians. In the real world, however, mishandling of samples can occur. These data indicate that varying the amount of PBS in the vials or delaying the addition of the PBS to the sample vials did not affect the outcome when testing BVDV-positive or negative ear-notch samples when using ACE. However, adding 6 mL or more PBS to the sample vial resulted in a lower S/P ratio for BVDV-positive ear-notch samples when utilizing the ACE. Despite this, samples used in this study were all strongly positive. However, this does not necessarily address the issue of weak positive samples and how these testing methods would effect those results.

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Endnotes

[a]IDEXX HerdChek* BVDV Antigen/Serum Plus Test Kit, IDEXX Laboratories, Inc., Westbrook, ME
[b]SAS version 9.1, SAS Institute Inc., Cary, NC

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