PEER REVIEWED

Evaluation of FTA cards as a sample collection method for detection of BVDV in persistently infected beef cattle

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Abstract

The accuracy of test outcome is directly related to the quality of samples arriving at the diagnostic laboratory. However, in certain field situations sample storage conditions may be less than optimal. Flinders Technology Associates (FTA) cards are a sample collection and storage tool suitable for field conditions. For the evaluation of FTA cards, nasal swabs and EDTA blood samples were collected by traditional methods from a group of known bovine viral diarrhea virus (BVDV) persistently infected (PI) animals, as well as from animals in a herd never diagnosed with BVDV infection. Diagnostic performance parameters of the reverse transcription real-time PCR assay (RT-r PCR) were compared between whole-blood and nasal-swab samples collected onto FTA cards from the same animals taken at the same time. Sensitivity and specificity based on the dichotomous classification of BVDV-positive or negative were 100% for the samples collected using traditional methods, and for those collected onto FTA cards. Real-time PCR cycle threshold values demonstrated a lower analytic sensitivity for samples collected onto FTA cards, although samples from FTA cards correctly identified all PI animals tested. One animal from the PI group tested BVDV-positive only in the nasal specimen, suggesting the positive resulted from contact exposure rather than from systemic viral production. To further evaluate the potential of the FTA cards, a subset of BVDV-positive samples (n=29) were sub-typed by direct sequencing of the BVDV qRT PCR products obtained from the case material. BVDV-1 was detected in 75.9% (22/29) of cases, BVDV-2 in 13.8% (4/29) of cases, and both serotypes were simultaneously detected in the remaining 10.3% (3/29) cases. Time savings associated with use of FTA cards for sample collection was calculated to be 23.5 seconds per blood sample. The FTA cards provide a method for diagnostic sample handling that helps overcome sample quality issues associated with improper storage conditions and shipping concerns.

Key words: bovine viral diarrhea virus, BVDV, FTA cards, persistently infected animals, PI animals, nasal swabs

Résumé

La précision des résultats d'un test est directement reliée à la qualité des échantillons soumis au laboratoire de diagnostic. L'entreposage des échantillons dans certaines conditions sur le terrain n'est pas toujours optimal. Les cartes de *Flinders Technology Associates* (FTA) sont des outils pour la cueillette et l'entreposage des échantillons adaptés aux conditions sur le terrain. Afin d'évaluer les cartes FTA, des écouvillons nasaux et des échantillons sanguins EDTA ont été recueillis par des méthodes traditionnelles chez des animaux immunotolérants infectés avec le virus de la diarrhée virale bovine (BVDV) de même que chez des animaux jamais diagnostiqués avec cette infection dans un troupeau. Les paramètres de performance diagnostic d'un essai à transcription inverse en temps réel ont été comparés pour les écouvillons nasaux et les échantillons de sang total recueillis sur les cartes FTA chez les mêmes animaux au même moment. La sensibilité et la spécificité, basées sur le statut BVDV positif ou négatif, étaient toutes deux de 100% pour les échantillons recueillis avec les méthodes traditionnelles et pour ceux recueillis sur les cartes FTA. Les valeurs seuils de la PCR en temps réel avaient une sensibilité analytique moindre chez les échantillons des cartes FTA bien que les échantillons des cartes FTA ont identifié correctement tous les animaux immunotolérants. Un animal du groupe immunotolérant a testé positif pour le BVDV seulement avec l'écouvillon nasal, suggérant que le résultat positif provenait d'une exposition de contact plutôt que d'une production virale systémique. Afin d'évaluer plus à fond les cartes FTA, un sous-ensemble des échantillons positifs au BVDV (n=29) ont été classés en sérotypes par le séquençage direct des produits BVDV dérivés du PCR en temps réel obtenus du

matériel des cas. Le sérotype BVDV-1 a été détecté dans 75.9% (22/29) des cas, le BVDV-2 dans 13.8% (4/29) des cas et les deux sérotypes étaient présents dans les derniers 10.3% (3/29) des cas. Les gains en temps associés avec l'utilisation des cartes FTA ont été évalués à 23.5 secondes par échantillon de sang. Les cartes FTA offrent une méthode pour la manipulation des échantillons de diagnostic qui permet d'éviter les problèmes de qualité des échantillons lorsqu'il y a des doutes concernant l'entreposage et le transport des échantillons.

Introduction

Bovine viral diarrhea virus (BVDV) causes one of the most important diseases affecting cloven hoofed species worldwide.[1,8,10-13,17,18,21] Early and accurate detection of cattle persistently infected (PI) with the virus is critical for prevention and control of the disease. Sample quality is important for obtaining a reliable diagnosis. Currently, ear notches, EDTA blood, serum, and nasal swabs are common specimens used for BVDV PI detection,[9] all of which require that temperature be controlled during transport to the laboratory. In the beef industry, samples are often collected under field conditions where ensuring that temperature is controlled from sample collection through transport and arrival at the laboratory is difficult. Samples which are compromised when arriving at the laboratory are either discarded, omitted from testing, or tested with a disclaimer regarding the potential loss of sensitivity of the assay. An accurate diagnosis is necessary to accurately identify BVDV carriers and to properly implement management protocols on the farm or ranch to prevent spread of the virus.

Flinders Technology Associates (FTA) cards[a] were developed as an alternative method of transport and storage of infectious samples for molecular diagnostics. The FTA cards contain a proprietary substrate which lyses cells and inactivates infectious agents, such as viruses and bacteria, while preserving nucleic acids.[5,16] Viral samples collected on FTA cards are rendered non-viable, making them useless for viral isolation or propagation. As a result, the samples do not pose a biohazard threat to humans or the environment. Viral agents, such as avian influenza virus, African swine fever virus, foot-and-mouth disease virus, and porcine reproductive and respiratory syndrome virus, have been safely collected using FTA cards.[4,14-16] Biological specimens stabilized on FTA cards can be mailed in regular envelopes using standard mail. To date, most studies using this technology have been performed using experimentally infected animals in controlled study environments, and have demonstrated excellent performance of FTA cards.[4,15,16] One manuscript describes the detection of BVDV in blood samples on a limited number of archived samples; however, testing was performed using conventional PCR technology and nasal swabs were not evaluated.[20] In the present study, several different parameters are evaluated to determine the suitability of the card for collection of samples and detection of BVDV PI animals in field settings. First, a comparison between blood samples collected in EDTA tubes and blood samples embedded on FTA cards was performed. Second, nasal fluids collected onto FTA cards were compared to the corresponding blood-sample test results to evaluate FTA cards as an easier sample collection and storage method for the detection of BVDV PI animals under field situations. Additionally, time needed to collect samples was monitored and compared to blood and nasal sampling methods. Lastly, genotyping of the BVDV directly from the diagnostic case material was performed on a subset of samples.

Material and Methods

Animals

Seventy previously tested, persistently infected feeder cattle housed together in a herd setting were sampled for this study. Animals were confirmed to be PI with BVDV by using the commercial antigen capture ELISA test kit[b], immunohistochemistry using anti-BVDV monoclonal antibody 3.12 Fl[c], RT-PCR, and virus isolation, as tested for a previous study.[9,10] During a routine health scan, animals were processed through a chute as part of herd monitoring, and samples were collected by trained personnel. Additionally, 22 weaned beef calves from a university owned cow-calf herd enrolled in a BVDV control program were sampled in a similar manner, no BVDV PI cases were ever diagnosed in this herd.

Blood samples were collected by jugular venipuncture. Blood was dripped out of the needle sufficient to fill a pre-marked circle (diameter 1 inch or 2.5 cm) on an FTA card or was collected into standard EDTA tubes. Nasal fluids were collected using a Dacron swab which was immediately rolled over the prescribed circle on the FTA card, thereby transferring nasal fluids from the swab to the card. Additionally, a freshly collected nasal swab was placed directly into a 15 mL conical tube with 3 mL of transport fluid. The time required to collect each sample type was recorded for the negative herd. The FTA cards were air dried and shipped overnight at ambient temperature, while EDTA blood samples were shipped to the testing laboratory overnight on wet ice at 39.2°F (4°C), the temperature typically recommended when shipping specimens for BVDV molecular diagnostic testing.

Nucleic Acid Extraction

RNA extraction from blood samples was performed using the MagMax Microarray kit[d], and swabs were pro-

cessed using the MagMax 96 kit[e], following the manufacturer's recommendations. Extracted RNA was eluted in 50µl of buffer. The FTA cards were processed as follows: a 5 mm circle was punched out of the card and 100µl of rapid extraction solution[f] was added. Residual nucleic acids were removed from the punch by twice punching a clean FTA card as recommended by the manufacturer[a]. After adding the rapid extraction solution, samples were further processed using the MagMax 96 kit, following the manufacturer's recommendations.

Real-time PCR

Extracted RNA was tested using a commercially available BVDV PCR kit[g] as a screening assay utilizing a 96-well, real-time PCR platform[h]. The assay includes an internal control (Xeno) for monitoring[i]. The PCR assay is USDA licensed, and detects BVDV strains by targeting the highly conserved 5' UTR region of BVDV. Results are expressed in cycle threshold values (Ct values), giving the number of cycles until the sample crosses the threshold between a negative and positive result. In general, a low Ct value indicates a higher virus concentration, whereas a higher Ct value suggests a lower virus concentration. BVDV genotyping was performed on the previously extracted RNA as described by Weinstock et al.[2]

Statistical Analysis

Data were analyzed using a commercially available software program[j] for basic statistical analysis. Bland Altman plots were used to demonstrate agreement between EDTA blood, and both FTA sample sets were prepared with the MedCalc Software program[k]. For purposes of this study, the BVDV results generated from Rt-r PCR of EDTA blood were considered to be the gold standard for comparison purposes. Additionally, the kappa coefficient[6,7] was calculated from dichotomous results for detection of BVDV. Kappa coefficients were considered "excellent" when above 0.81, "fair" when from 0.4 to 0.81, and "poor" when below 0.4.[19]

Results

Internal controls, based on cycle threshold (Ct) values for all 3 sample collection types for both BVDV-positive and BVDV-negative samples, performed consistently as demonstrated by the small standard deviation (Table 1). Extraction failure, identified by the inability to detect the internal control, was found in 1% of EDTA blood samples (1/92) and 3.3% of FTA blood samples (3/92), triggering a re-extraction of the sample. No failure of extraction was noted in the FTA nasal swab set.

From a total of 92 matched blood samples collected from known BVDV PI cattle (n=70) and presumed negative animals, 98.6% (69/70) of the previously positive samples tested positive in both the EDTA and FTA sample collections (Table 2). The Ct values ranged from 12.08 to 27.76, with a median of 18.64 Ct in the EDTA blood samples. The FTA card Ct values for blood samples ranged from 22.90 to 33.92 Ct, with a median Ct value of 26.9 Ct. One of the previously BVDV PI-positive animals tested negative in the blood sample using both sample collection techniques. No BVDV was detected on any of the presumed negative samples when using either sample storage method (100%, n=22).

Ninety-two nasal swab samples were collected onto FTA cards. All 70 of the previously positive animals tested positive for BVDV when using the nasal-swab samples. Of 70 samples from BVDV PI-positive animals, 69 were positive in all 3 of sample collection methods: EDTA blood, blood FTA, and nasal-swab FTA. One animal tested negative when using both blood sample collection techniques, but positive when using the nasal-swab FTA sample. The Ct values for the 70 BVDV-positive nasal-swab FTA samples ranged from 19.3 to 36.68, with a median of 24.54 Ct. Dichotomous test results and mean Ct for each sample type are listed in Table 2. No BVDV was detected in nasal swabs from animals in the negative herd.

Bland-Altman Plots,[3] a graphical representation of agreement between 2 assays, were constructed to determine the agreement between the 2 sample collection methods. Using MedCalc, Bland-Altman plots of Ct for

Table 1. Average cycle threshold values, standard deviation of Ct value, and number of samples requiring re-testing for the internal control for each sample type.

	Mean Ct value	Standard deviation	Failure to detect
EDTA blood	30.86 Ct	1.03 Ct	1
FTA card blood	31.38 Ct	0.51 Ct	3
FTA card nasal swab	30.73 Ct	0.56 Ct	0

Table 2. Dichotomous results of BVDV testing and average Ct value for each sample type.

PCR result	Positive	Negative	Mean Ct values
EDTA blood	69	23	18.5
FTA card blood	69	23	26.8
FTA card nasal swab	70	22	25.8

EDTA blood compared to the Ct for FTA blood for positive animals (Figure 1) and Ct for EDTA blood and nasal swabs were created (Figure 2). The widths of the 95% agreement interval were 7.31 and 14.81 for FTA blood and nasal swabs, respectively, indicating good agreement between the PCR results from the 2 methods of blood collection for BVDV testing. All samples taken from a subset of 30 animals previously identified as BVDV PI (a total of 88 samples including 29 EDTA, 29 FTA blood, and 30 FTA nasal swabs) were subjected to comparative genotyping. This subset included the animal that tested positive for BVDV only from the nasal swab; therefore, all calculations were performed with a denominator of 29. Results varied between the Ct values, depending on the sample collection method (Table 2). A total of 23/29 (79.3%) samples showed perfect agreement in the genotyping assay based on dichotomous data analysis. Of the 29 samples collected in EDTA blood from PI animals, the most prevalent genotype was BVDV-1 (22/29, 75.9%), while 4 animals (13.8%) tested positive for BVDV-2 and 3 samples (10.3%) tested positive for both BVDV genotypes. There was disagreement between 3 blood samples collected on FTA cards compared to the gold-standard EDTA blood. Two samples were genotyped as BVDV-2 using EDTA blood, but the virus was not detected using the FTA blood-card samples. The third sample yielded a dual infection according to the gold standard; however, only

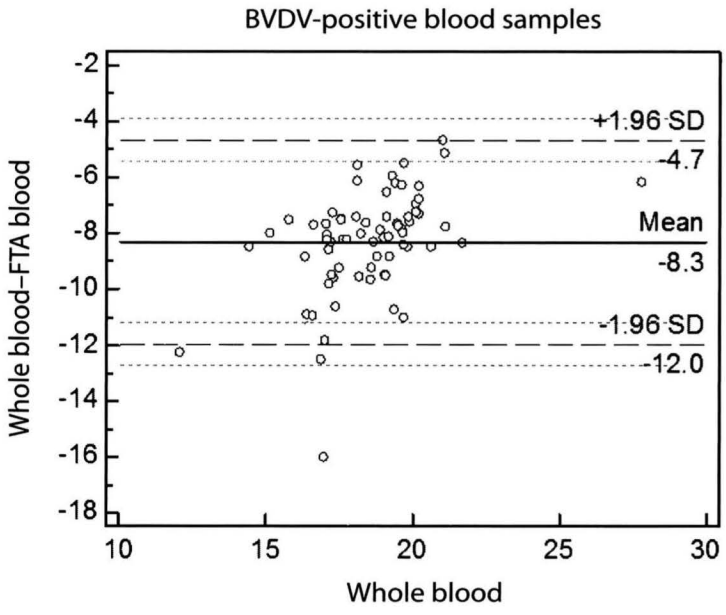

Figure 1. Bland Altman Plot demonstrating the difference between the Ct of whole blood and blood collected on FTA cards compared to the Ct of whole blood collected for RT PCR. Samples were collected from 69 BVDV PI beef cattle from the central United States.

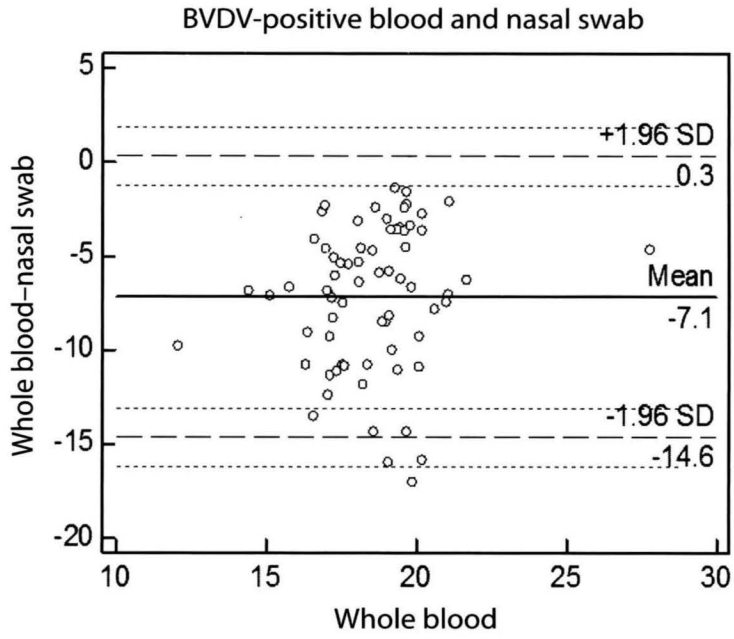

Figure 2. Bland Altman Plot demonstrating the difference between the Ct of whole blood and nasal swabs collected on FTA cards to the Ct of whole blood RT-PCR. Samples were collected from 70 BVDV PI beef cattle from the central United States.

BVDV-1 was detected from the FTA blood sample. Overall agreement was 100% (29/29) for BVDV-1 detection, and 93.1% (27/29) for BVDV-2 detection. Comparison between the 2 different sample types, EDTA blood and nasal swabs collected on FTA card, was 93.1% (27/29) for BVDV-1 detection and 96.6% (28/29) for detection of BVDV-2. Two of the 3 incompatibilities for BVDV-1 were due to the additional detection of the BVDV-1 genotype in nasal swabs, whereas the gold standard had only confirmed the presence of BVDV-2. The third discrepancy was the failure to detect a BVDV-1 genotype from nasal swabs collected on FTA cards, whereas the virus was detected using the gold standard. This particular animal did not shed detectable amounts of either BVDV genotype in nasal secretions. One animal tested positive only on the nasal swab, with no virus detected in the corresponding blood sample. The genotype found in the nasal-swab sample was BVDV-1 (Table 3).

The kappa coefficient,[6,7] estimating the diagnostic agreement between the different sample types, was calculated at K=1.0 for comparison between EDTA blood and FTA blood, and showed a value of K=0.97 for comparison between EDTA blood and FTA nasal swab. Both kappa values showed excellent agreement between EDTA blood and the 2 FTA sampling methods as a BVDV PI screening test.

Table 3. Genotyping results indicating the number of samples in which each BVDV genotype occurred by sample type.

	EDTA blood	FTA blood 5mm punch (repeated)	FTA nasal swab 5mm punch
BVDV-1	22	22 (23)	23
BVDV-2	4	3 (3)	3
BVDV-1 & BVDV-2	3	1 (1)	3
Total	29	26 (27)	29

When collecting samples from the negative herd, the average time needed for sample acquisition was 27.4 seconds (range 9 to 102 sec) per sample for collecting blood from the jugular vein into an evacuated blood tube or onto an FTA card. In general, 2 people were necessary to complete this procedure. Nasal swab collection and rolling of the Dacron swab onto the FTA card was performed in 3.9 seconds (range 2 to 7 seconds) per sample, and was easily completed by 1 person. The difference in time for sample collection for blood versus nasal swabs was statistically significant ($P < 0.005$).

Discussion

This study evaluated different methods for collection of samples to test for BVDV PI animals in field settings. FTA cards were evaluated for their potential to provide a practical and less expensive way to collect and submit high quality samples to the laboratory. The stability of the viral RNA once air dried on the filter card, as well as the ease and decreased cost of mailing in a regular envelope, seem especially beneficial when working in remote locations where access to specialized shipping containers, wet or dry ice, and routine courier services is limited. All BVDV PI animals were detected by both sampling methods; however, the Ct values resulting from the FTA card were higher. The 8.4 Ct difference is estimated as 2 to 3 logs lower detection limit using the FTA card, using the convention of 3.3 Ct as an estimate of 1 log difference in RT-r PCR detection. One log of that difference can be accounted for by the lower starting volume of blood from the FTA card punch utilized in the pre-PCR nucleic acid extraction process.

The same phenomenon was seen comparing standard dilutions series. Both methods demonstrated a working range of 10^5 dilutions, while the Ct values recovered from the FTA dilution series were approximately 6 Ct higher (~2 logs) compared to the EDTA blood extraction method (Table 4). Persistently infected cattle are thought to continuously circulate higher titers of virus in their blood stream than normal cattle, therefore we believe that the lower detection sensitivity of the FTA cards will not negatively impact the dichotomous test outcome. Although further studies involving both BVDV PI animals and animals transiently infected with BVDV are required, all PI animals were successfully detected in this study.

In the second comparison, nasal swabs collected on FTA cards were compared to the matching EDTA blood results to evaluate the easier sampling route under field situations. Nasal swab samples are easily taken by 1 trained person, whereas 2 people, 1 of them specifically trained on blood sampling procedures, were usually needed for collection of blood from the jugular vein. As found with the FTA blood samples, the Ct values from the FTA nasal swabs were on average 7.3 Ct higher than EDTA blood. Corresponding to a decreased sensitivity of approximately 2.2 logs of virus. This might be due to a lower concentration of virus in nasal swabs compared to the virus concentration circulating in the blood, the lower starting volume used for FTA card samples, or due to lower extraction efficiency between EDTA and FTA cards. All PI animals were detected using FTA nasal-swab testing, assuring the FTA card sampling method is a valuable tool for the producer or veterinarian under difficult field conditions.

One known positive animal tested negative when using both blood sampling methods, but the nasal swab sample tested positive. Earlier, this animal tested positive for BVDV when using the antigen capture ELISA

Table 4. Dilution series indicating average Ct values for 3 independent runs of each dilution for EDTA blood, FTA blood, and the internal control (Xeno).

Dilution series preliminary comparison				
BVDV dilution	**EDTA**	**Xeno**	**FTA**	**Xeno**
10	18.16	29.60	26.33	30.39
10^2	22.40	30.37	30.52	31.00
10^3	25.50	31.02	33.62	31.06
10^4	28.87	31.84	36.66	31.15
10^5	32.30	32.03	38.76	31.57

test, therefore it is possible that the animal was transiently infected with BVDV rather than PI when tested earlier. Because this non-PI animal was housed with 69 animals shedding BVDV, it is possible that detection of BVDV in the nasal swab sample was due to exposure to BVDV in the PI calves or the environment. This highlights the importance of confirming BVDV PI animals by submitting a second blood or tissue sample from PI suspects at least 2 weeks following the initial test to confirm PI status.

In addition to the cost and number of personnel needed for sampling, time required for the sample collection was considered and compared between blood and nasal collection methods. Nasal swab collection was faster, less expensive, and required fewer personnel compared to traditional blood collection. A nasal swab collection could be easily completed alone by an owner or ranch hand.

Lastly, virus genotyping directly from diagnostic case material was evaluated on a subset of samples. Some discrepancies among the 3 sample types were observed in the genotyping assay, and further investigation is warranted. Additional serotypes detected in nasal swabs versus blood may result from testing a transiently infected animal which has subsequently recovered from infection, or indicate environmental exposure to multiple viral types.

Conclusions

A comparison of traditionally collected blood samples and samples collected onto and shipped using FTA cards to detect BVDV PI animals showed comparable performance based on calculated kappa values. Comparison of the numerical Ct values showed overall lower detection sensitivity for FTA cards when compared to traditionally collected blood samples. Nonetheless, the dichotomous (positive/negative) results for detection of BVDV PI animals were identical. Congruency between the results of samples tested using EDTA blood and samples tested using FTA blood and FTA nasal swabs indicate that FTA cards are a valid method for blood storage and transport for BVDV screening when using PCR technology. Further research is needed to determine if blood collected using this method can be used to differentiate between animals which are transiently infected and those which are persistently infected with BVDV.

Endnotes

[a]Whatman FTA cards, GE Healthcare Life Sciences, Piscataway, NJ
[b]IDEXX Laboratories, Westbrook, ME
[c]Central States Testing® NS23, Central States Testing®, Sublette, KS
[d]MagMax™-96 Viral RNA Isolation Kit (#AM1836), Life Technologies, Grand Island, NY
[e]MagMAX™-96 for Microarrays Total RNA Isolation Kit (#AM1839) Life Technologies, Grand Island, NY
[f]RNA Rapid Extraction Solution (#AM9775), Life Technologies, Grand Island, NY
[g]VetMAX™-Gold SIV Detection Kit (#4415200), Life Technologies, Grand Island, NY
[h]Applied Biosystems® 7500 Fast Real-Time PCR System, Life Technologies, Grand Island, NY
[i]TaqMan® BVDV and Xeno RNA Controls, Life Technologies, Grand Island, NY
[j]Excel 2010, Microsoft Corporation, Redmond, WA
[k]MedCalc Software program, Mariakerke, Belgium

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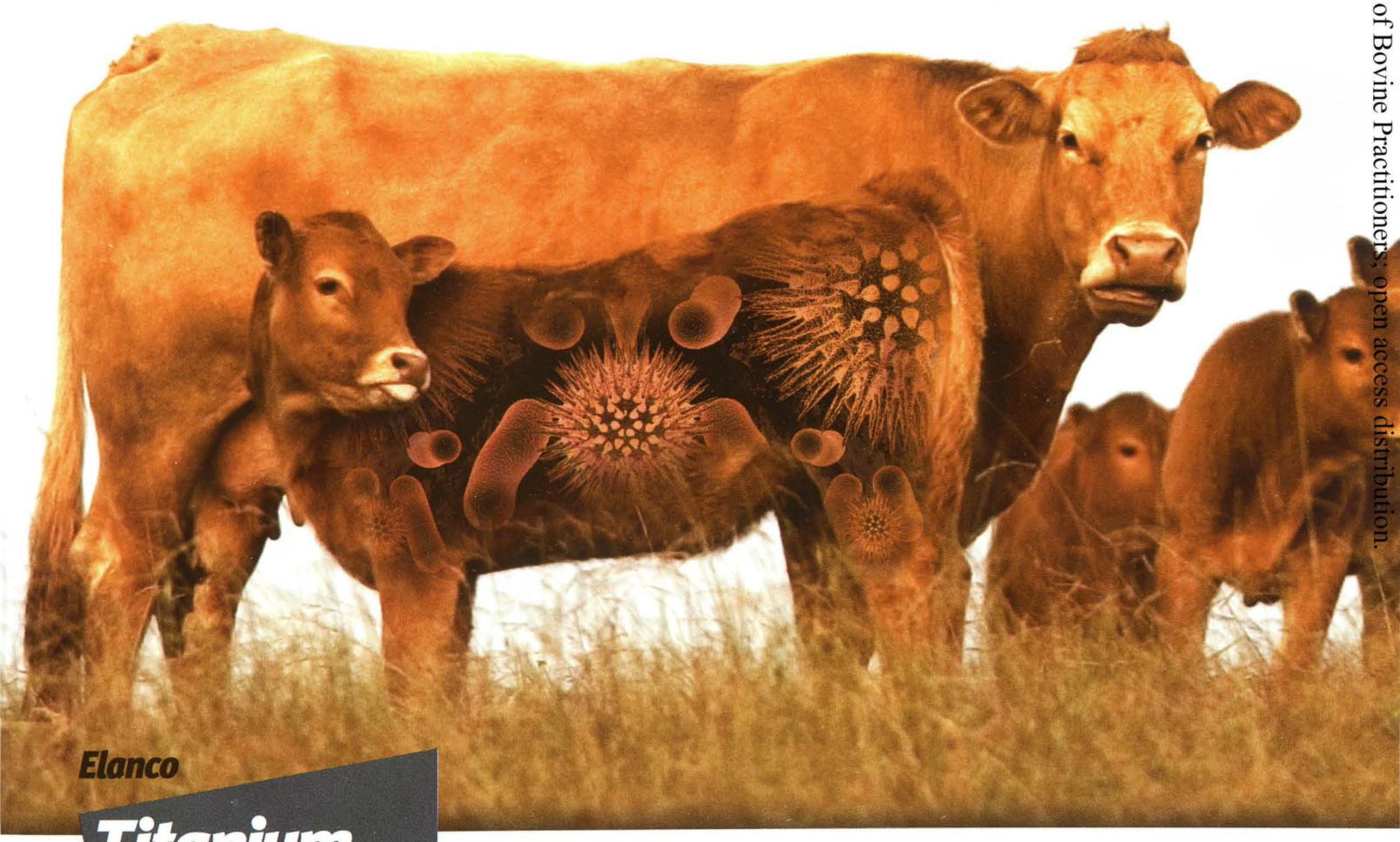

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  - Offers the convenience of a single vaccine at every stage of production
  - Contains a research-proven *M. haemolytica* leukotoxoid and other antigens to stimulate an immune response to BRD-causing viruses and bacteria
  - Includes a low-reactive, water-soluble adjuvant, and is proven safe for cattle as young as 2 months of age
  - Its low-volume, subcutaneous administration meets Beef Quality Assurance℠ (BQA) guidelines

The Titanium line of vaccines is a brand producers trust — the brand that brought you the first type 2 BVD modified-live virus (MLV) vaccine.

The label contains complete use information, including cautions and warnings. Always read, understand and follow the label and use directions. Do not vaccinate within 21 days of slaughter.

Indications:
For the vaccination of healthy cattle, 60 days of age or older.

Directions and dosage:
Rehydrate the modified live virus vaccine with accompanying bottle of liquid bacterin-toxoid and shake well. Inject 2 mL subcutaneously, using aseptic technique, followed by a second dose of monovalent bovine respiratory syncytial virus vaccine (Titanium® BRSV) and *M. haemolytica* and *P. multocida* vaccine (PULMO-GUARD® PH-M) to be given 14 to 28 days after the first dose. Annual revaccination is recommended. Calves vaccinated before weaning should be revaccinated 30 days after weaning when the possible influence of maternal antibodies is decreased.

Storage:
Store at 35° to 46° F (2° to 8° C). DO NOT FREEZE. Shake well before using. Use entire contents when first opened. Do not vaccinate within 21 days before slaughter. Burn container and all unused contents. Contains thimerosal as a preservative.

Product codes:
10 dose: AV418584GAM 50 dose: AV418584TAM

Titanium 5 + PH-M delivers a broad immune response against the bacteria and viruses most associated with BRD in a single vaccine for cattle as young as 2 months of age.[1-4]

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[1]Demonstration of the compatibility of components between APHIS product codes 1181.20 (Establishment 213) and G935.04 (Establishment 315) APHIS product code 45B9.20. Study No. 2010-01 Rev. 1.
[2]Efficacy study for BRSV fraction to demonstrate compatibility of the BRSV component in APHIS product code: 45B9.20 (unlicensed). Study No. 2011-05 Rev. 1.
[3]Milliken, G. A. 2013. Mannheimia haemolytica efficacy studies demonstrating the absence of excessive interference of Titanium products with the Mannheimia haemolytica-Pasteurella multocida bacterin-toxoid.
[4]Porter, M. 2013. Field safety evaluation of bovine rhinotracheitis-virus diarrhea-parainfluenza$_3$-respiratory syncytial virus vaccine, modified live virus, APHIS product code 1181.20 (establishment 213) in combination with Mannheimia haemolytica-Pasteurella multocida bacterin-toxoid, APHIS product code: G935.04 (establishment 315). Protocol No. 2011-01, Rev. 02.

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