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J Vet Diagn Invest 8:99-105 (1996)

Evaluation of two antigen-capture ELISAs using polyclonal or monoclonal antibodies for the detection of bovine coronavirus

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Bovine coronavirus (BCV) is recognized as a common causative agent of neonatal calf diarrhea.²¹ It has also been incriminated by several researchers from around the world as a causative agent for winter dysentery (WD), an acute diarrheal disease of adult cattle.^{1,8,17,20,22,25,29,31} Review articles concerning BCV⁴ and the immunology of coronaviruses have recently been published. Currently, the diagnosis of BCV infection is usually made by identifying the virus in feces.⁴ Because isolation of BCV in cell culture is difficult, this method is rarely employed as a diagnostic test; electron microscopy (EM) is often used for the identification of BCV in feces.^{2,10} Although the intact virion of BCV is fairly characteristic in appearance, it is not uncommon for the identifying surface projections of the virus to be lost during sample preparation or storage, making it more difficult to properly identify virus particles by EM. Immunoelectron microscopy (IEM), utilizing specific antibodies against BCV, is used to increase the sensitivity and specificity of EM.^{4,10} Enzyme-linked immunosorbent assays (ELISAs) have been described

for the detection of BCV antigen in feces, but these assays have lacked sensitivity when compared with similar assays for other enteric pathogens.^{6,7,10,15,23} The use of monoclonal antibodies rather than polyclonal antibodies has increased the sensitivity and specificity of BCV ELISAs.^{7,23} ELISAs offer an advantage over EM and IEM of being able to rapidly evaluate large numbers of samples. This advantage is important for epidemiologists conducting large surveys and diagnosticians who must examine many submissions.

In this report, we describe the development and evaluation of 2 ELISAs for detection of BCV antigen in feces. In 1, polyclonal antibodies were used for antigen capture (PA-CELISA); the other was identical except for the use of monoclonal antibodies (MABs) (MACELISA). We also present new information related to evaluation of the 2 assays for detection of both calf and WD BCV strains and the sensitivity and specificity obtained with the MACELISA.

Nine strains of BCV adapted to cell culture in human rectal tumor (HRT-18) cells as previously described³ were used to evaluate the ELISAs. Of the 9 strains evaluated, 2 (Mebus, DB2) were isolated from diarrheic calves and 7 (DBA, SD, BE, BM, AW, TS, CN) were isolated from adult cows clinically affected with WD.^{3,14,28}

Sixty reference fecal samples were collected from gnotobiotic calves or field cases of neonatal calf scours for which the BCV infection status was determined by EM, IEM, or immunofluorescence. These samples were diluted 1:25 in phosphate-buffered saline (PBS) and centrifuged (850 x g,

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Received for publication November 2, 1994.

20 minutes), and the supernatant was saved for ELISA testing. Of these 60 fecal samples, 36 BCV-positive samples were obtained from 16 gnotobiotic calves experimentally infected with BCV and from 20 field cases of calf diarrhea. All 20 of the BCV-positive field samples were from calves concurrently infected with rotavirus, as determined by EM. Twenty-four BCV-negative fecal samples were obtained from 23 field cases of calf diarrhea and 1 gnotobiotic calf. All 23 BCV-negative field samples were from diarrheic calves infected with 1 or more neonatal diarrheal pathogens (rotavirus, $n = 16$; Breda virus, $n = 1$; cryptosporidia, $n = 7$). In addition to the 60 fecal samples described, 2 Breda virus-positive fecal samples (by IEM) from gnotobiotic calves infected with Breda virus were tested to determine the ability of the MACELISA to distinguish this morphologically similar but antigenically distinct virus from BCV. Fecal samples and cell culture passaged virus aliquots were stored at -70 C until prepared for testing.

Hyperimmune serum prepared in a gnotobiotic calf in response to the Mebus strain of BCV was used for the polyclonal antibody positive coating.¹⁰ This serum had a virus neutralization (VN) antibody titer to Mebus BCV of 1:32,000. For negative coating, serum from a newborn gnotobiotic calf free of BCV antibodies was used. Both sera were diluted 1:2 with glycerol and stored at -20 C .

MAbs against the virulent DB2 strain of BCV were prepared in mice as previously described.^{12,32} Antibody titers of the MAbs to the Mebus strain of BCV were determined by VN and indirect immunofluorescence assays.^{12,32} The BCV protein specificity of the MAbs was determined by western blot assay against the Mebus strain of BCV (Heckert and Saif, unpublished). Ten MAbs with the highest antibody titers to BCV, as determined by indirect cell culture immunofluorescence assay,¹² were chosen for further evaluation as positive coating for ELISA. The MAbs were evaluated as diluted ascitic fluid in the ELISA first individually against cell culture-adapted strains of BCV and then as pools of 3, with each pool evaluated containing MAbs directed against 3 BCV structural proteins (nucleocapsid [N], spike protein [S], hemagglutinin esterase [HE]). Titers to DBA, SD, and Mebus strains of BCV, determined by indirect immunofluorescent antibody tests, were 1:25,600, 1:51,200, and 1:25,600, respectively, for the MAb directed against the HE viral protein; 1:51,200 for all 3 viral strains for the MAb directed against the S viral protein; and $> 1:102,400$ for all 3 strains for the MAb directed against the N viral protein. Negative coating was diluted ascitic fluid from mice inoculated with SP2/0 mouse myeloma cells.

Immunoelectron microscopy was conducted using procedures similar to those described previously.¹⁹ Fecal samples were diluted 1:5 in PBS, sonicated, and clarified at 4 C , and the supernatants were filtered through $0.45\text{-}\mu\text{m}$ syringe filters.^a Diluted gnotobiotic calf anti-BCV serum was added to the supernatants and incubated overnight at 4 C . The immune complexes were pelleted by centrifugation ($69,000 \times g$, 35 minutes, 4 C) and then resuspended in sterile distilled H_2O (dH_2O), repelleted as above, suspended in $50\ \mu\text{l}$ dH_2O , and vortexed. For EM, 1 drop of 3% PTA ($\text{pH } 7.0$) was added, and 1 drop of the suspension placed on formvar-coated carbonized copper grids.

The ELISAs described were indirect double antibody sandwich antigen-capture assays. In the PACELISA, paired rows of a 96-well microtiter plate^b were coated with $100\ \mu\text{l}$ /well of a 1:4,000 dilution of polyclonal gnotobiotic calf hyperimmune anti-BCV serum (B6429, positive coating) or a 1:4,000 dilution of BCV antibody-negative serum (negative coating) in carbonate-bicarbonate buffer. Plates were incubated at 4 C overnight or stored at 4 C for up to 7 days. After washing, $200\ \mu\text{l}$ of 5% nonfat dry milk in PBS was added to each well as a blocking step to minimize nonspecific binding, and the plates were incubated for 2 hours at 25 C . The plates were washed, and $100\ \mu\text{l}$ of the test samples (cell culture virus or 1:25 dilutions of feces) were placed in paired wells of BCV antibody-positive and -negative serum coating. Plates were incubated at 4 C for 16 hours. The plates were washed, and $100\ \mu\text{l}$ of guinea pig anti-BCV (Mebus) serum diluted 1:4,000 in PBS/0.05% Tween 20/2% bovine serum albumin was added to each well. The plates were incubated 1 hour at 25 C and washed, and $100\ \mu\text{l}$ of a 1:4,000 dilution of sheep anti-guinea pig peroxidase-conjugated antibody^c in PBS/2% bovine serum albumin was added to each well. Plates were incubated 1 hour at 25 C and washed. One hundred microliters of the chromogen substrate, a 1:1,000 solution of H_2O_2 and 2,2'-azino-bis(3-ethyl-benzthiazoline)sulfonic acid in 0.1 M sodium citrate was applied to each well. After 20 minutes, the color reaction was stopped by the addition of $50\ \mu\text{l}$ of 5% sodium dodecyl sulfate per well. The absorbance value of each well was read at a wavelength of 414 nm with a computer-linked ELISA plate reader,^d and the readings were saved as ASCII files.^e

At each washing, the plates were rinsed 5 times with PBS/0.05% Tween 20. Plates were sealed with ELISA plate tape during each incubation step. A fecal sample from a gnotobiotic calf infected with BCV and determined positive for virus by IEM was used on each plate as a positive control. The negative control fecal sample was from a diarrheic field calf determined negative for BCV by IEM.

The MACELISA procedure was identical to the PACELISA except MAbs to BCV were substituted for the polyclonal antibodies for coating the plates. In the MACELISA, a pool of 3 MAbs directed against 3 structural BCV proteins (N, S, HE) was used as positive coating. One hundred microliters of a 1:8,000 dilution of the 3 MAbs, used as mouse ascites fluids, was added to paired rows as positive coating. Similarly, $100\ \mu\text{l}$ of a 1:8,000 dilution of BCV antibody-negative mouse ascites fluids was added to paired rows as negative coating.

A spreadsheet program^f was used to calculate the ELISA value for each sample. The mathematical calculation of the ELISA value for each fecal sample was the average absorbance of the paired positive-antibody-coated wells minus the average absorbance of the paired negative-antibody-coated wells.

To determine the analytical sensitivity of each ELISA to detect BCV antigens, serial dilutions of 9 cell-culture-adapted strains of BCV were tested in both assays. The TCID_{50} of BCV in cell culture medium was determined by the Reed-Munch method, and $100\ \mu\text{l}$ of 4-fold serial dilutions of the cell culture BCV suspensions in PBS were applied to antibody-positive coated wells. The ELISA value used for de-

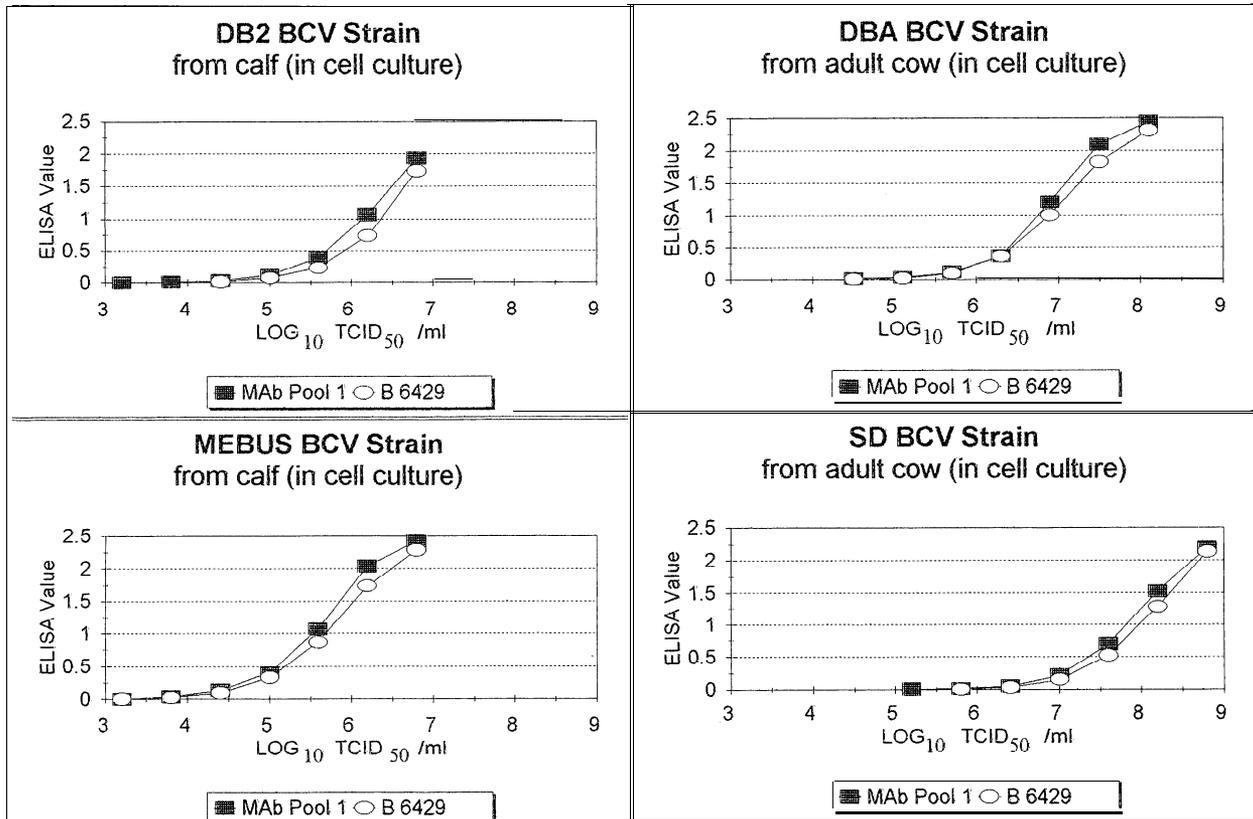


Figure 1. End point of virus titer detection by ELISA from 4 BCV strains in cell culture. The ELISA value for this comparison was calculated by subtracting the absorbance of PBS on positive coating from the absorbance of the cell culture virus diluted in PBS on positive coating. The positive coating was either a MAb pool (MAb pool 1) or gnotobiotic calf hyperimmune serum against BCV (B6429). An ELISA value of 0 is the value of the diluent (PBS) on positive coating; ELISA values >0.007 were considered positive.

detecting the cell culture BCV titer end point was defined as the absorbance value of virus diluted in PBS on positive coating minus the absorbance of PBS with no virus. The virus dilution at which the ELISA value was greater than an empirical 0.007 was determined to be the end point and was expressed as the minimum TCID₅₀ detectable.

For each assay, the frequency distribution of the ELISA values for 60 BCV-positive or -negative fecal samples previously described were calculated and graphed. From the frequency distribution data, the sensitivity and specificity at each ELISA value was calculated and graphed. The mathematical product of sensitivity \times specificity, termed efficiency, was calculated and graphed for each ELISA value to provide the probability of correct classification given a single sample of unknown status (Hancock, 1994, personal communication).^{9,27} Each ELISA cutoff value was optimized at the point of greatest efficiency. Using the cutoff value determined for each assay, the optimum combination of sensitivity and specificity of each assay was determined.

Fifty-six (33 BCV-positive samples and 23 BCV-negative samples) of the 60 reference fecal samples were tested using EM or IEM. Using the same previously determined ELISA cutoff values and the results from these 56 fecal samples, a kappa value was calculated for both the PACELISA and the MACELISA to measure the agreement of each ELISA with EM/IEM results.¹³

Both assays detected all 9 BCV strains from cell culture. Both assays detected each strain of cell culture virus at similar end points of virus titer (Fig. 1). There was up to a 2 log₁₀ difference in the analytical sensitivities among various strains with generally greater analytical sensitivity of both assays with the calf strains of BCV (DB-2, Mebus) than with the WD strains. The PACELISA had analytical sensitivities expressed in minimum TCID₅₀ detectable, for each strain as follows: DBA, 10^{5.1}; SD, 10^{5.8}; CN, 10^{4.9}; BE, 10^{4.4}; BM, 10^{5.5}; AW, 10^{5.5}; TS, 10^{3.9}; Mebus, 10^{3.8}; DB-2, 10^{4.4}. Similarly, the analytical sensitivities for MACELISA were DBA, 10^{4.5}; SD, 10^{5.8}; CN, 10^{4.9}; BE, 10^{4.4}; BM, 10^{4.9}; AW, 10^{4.9}; TS, 10^{3.9}; Mebus 10^{3.8}; and DB-2 10^{3.8}.

The frequency distributions of the values from MACELISA and PACELISA obtained from the 36 BCV-positive and 24 BCV-negative reference fecal samples are illustrated in Fig. 2. There was greater differentiation in the frequency distribution of values from positive and negative samples with the MACELISA than with the PACELISA (Fig. 2). Utilizing the calculations for efficiency, the optimum cutoff value for the PACELISA was 0.110 (Fig. 3A); the optimum cutoff value for the MACELISA was 0.030 (Fig. 3B). At these cutoff values for the calf fecal samples, the sensitivity of the PACELISA was 80.6% (95% confidence interval of 91.2-63.4%) and the specificity was 95.8% (99.8-76.9%); for the MACELISA the sensitivity was 97.2% (99.9-83.8%) and the

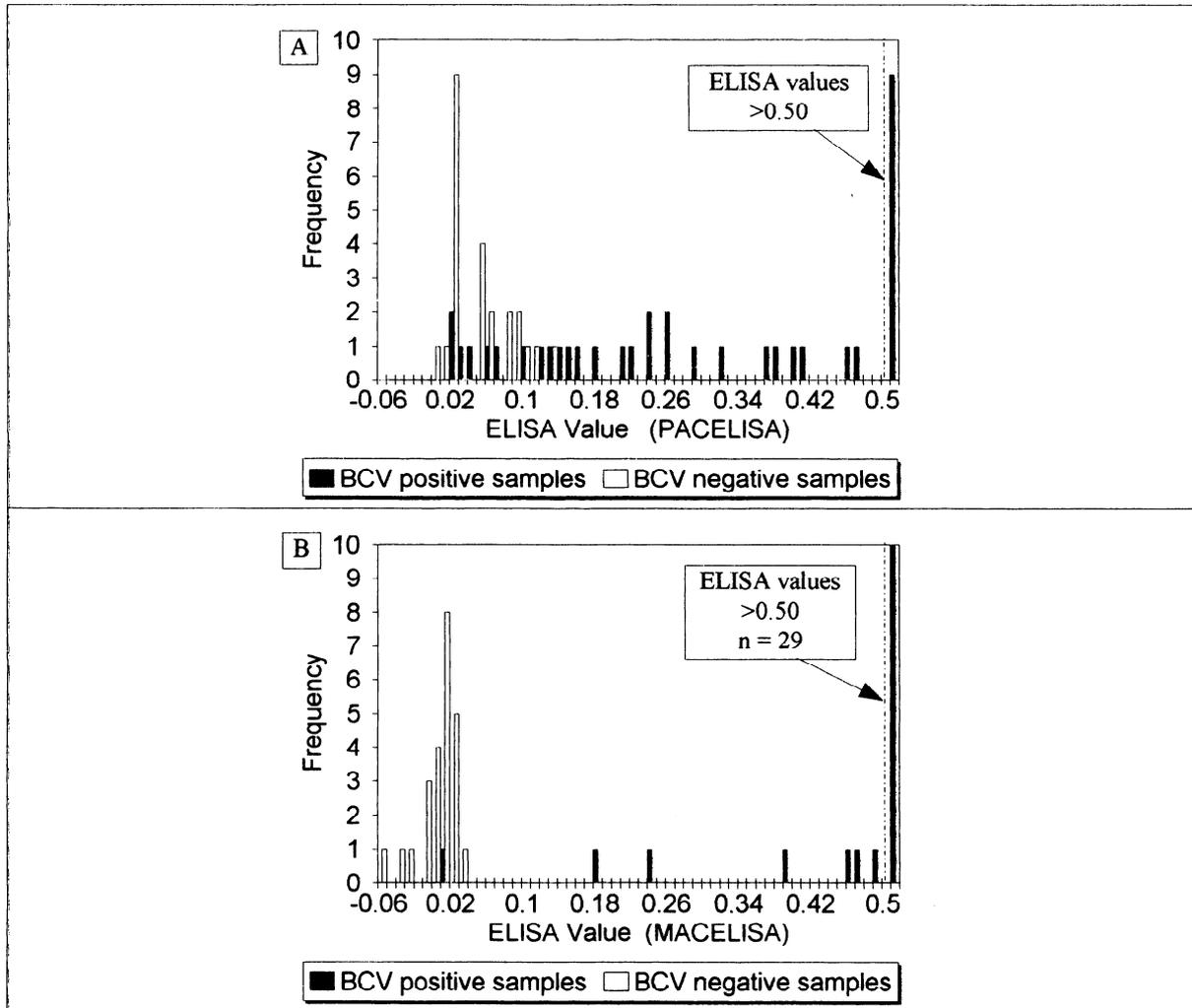


Figure 2. Frequency distribution of ELISA values obtained by BCV antigen-capture ELISA from 36 BCV-positive and 24 BCV-negative reference fecal samples. A. ELISA with polyclonal antibodies (PACELISA). B. ELISA with MAbs (MACELISA).

specificity was 100% (100/438.3%). The 2 additional Breda virus IEM-positive fecal samples from gnotobiotic calves were negative for BCV antigens when tested by MACELISA (values of 0.013 and 0.022).

Kappa values measuring agreement with EM/IEM results at the optimum cutoff value for MACELISA and PACELISA were 0.96 (95% confidence interval of 1.0489) and 0.75 (0.92-0.58), respectively, for the calf fecal samples.

Subtracting the background value (the nonspecific reaction occurring within the plates) from the signal left the ELISA value that represents the specific antigen-antibody reaction occurring within the wells. To determine the comparable end point of virus detection by both ELISAs, cell culture BCV was serially diluted with PBS. The background value in this case was considered to be the absorbance value of PBS on positive coating. By subtracting the absorbance value of PBS on the positive coating to calculate the ELISA value, we were able to compare the effectiveness of the 2 positive coatings for attracting antigen; positive ELISA values indicate the

detection of BCV antigen as compared with PBS with no virus.

Fecal samples present a more difficult challenge for the correct interpretation of ELISA results. Feces contain biologic and immunologic constituents that may affect the occurrence of nonspecific reactions,⁵ so that each fecal sample will have a unique background absorbance value associated with it. To minimize the effect of background, BCV antibody-negative coating was utilized with the assumption that the absorbance value obtained from the negative coating represents the nonspecific reactions that also take place on the positive coating. The ELISA value calculation we used for fecal samples essentially blanks each fecal sample for its own unique background.

It is not uncommon for biologic data to have non-Gaussian distributions.¹¹ The method of determining the optimum cutoff value employed uses a non-Gaussian method that relies on the frequency distribution of known BCV-positive and -negative fecal samples to determine the sensitivity and spec-

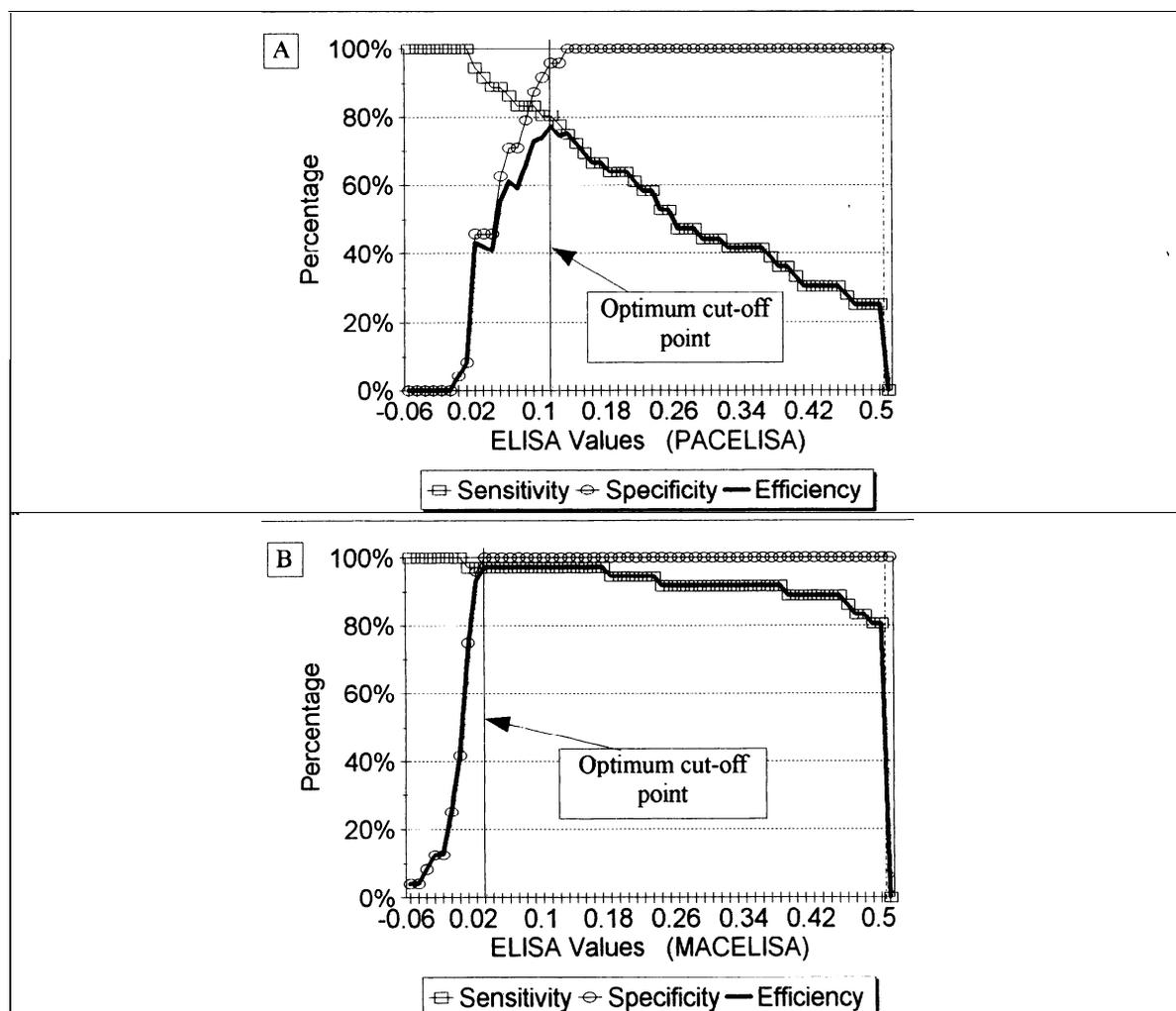


Figure 3. Sensitivity, specificity, and efficiency of BCV antigen-capture ELISAs illustrating calculation of the optimum cutoff values (vertical lines) to minimize errors (false positive and false negative). **A.** ELISA with polyclonal antibodies (PACELISA). **B.** ELISA with MAbs (MACELISA).

ificity of the assays at each ELISA value interval. At the point of maximum efficiency, the fewest errors, both false positive and false negative, are made. This point intuitively presents itself as the optimum point for the cutoff value assuming that the cost of either error is the same.

Both assays demonstrated excellent specificity. All of the BCV-positive and -negative reference samples from diarrheic field calves with other concurrent enteric infections. It is reasonable to expect that diagnostic fecal samples from calves with neonatal diarrhea also will have more than 1 pathogen present.^{16,24} Both tests exhibited the ability to identify BCV antigen in feces to the exclusion of other calf enteric pathogens. Breda virus is morphologically similar but antigenically distinct from BCV and has also been incriminated in WD outbreaks;²⁹ therefore, we used the MACELISA to test 2 fecal samples from gnotobiotic calves that contained Breda virus and were negative for BCV by IEM. These 2 samples were negative by MACELISA, indicating that the presence of Breda virus in field specimens did not give false-positive results.

The sensitivity and specificity of the MACELISA were not statistically different from those of the PACELISA. However, upon examination of the distribution of the values obtained by both assays using the same samples, the MACELISA appeared to more clearly differentiate BCV-positive samples from BCV-negative samples (Fig. 2). This clear distinction of negative and positive samples with the use of MAbs rather than polyclonal antibodies is probably a result of the advantages of specificity of binding and homogeneity. Thus, the problems of cross-reactivity of naturally occurring polyclonal antibodies are minimized.³³ Other advantages associated with the use of MAbs over polyclonal antibodies are that uniform reagents are consistently available, leading to reproducible results from assay to assay and laboratory to laboratory. The use of a MAb pool directed at different epitopes gives the theoretical advantage of increasing the affinity for the antigen over that of a single MAb, which can bind at only 1 site.³³

Evaluation of an assay by the calculation of sensitivity and specificity makes the assumption that the reference samples

have been diagnosed without error by an infallible reference assay; rarely can this assumption be met. An alternative method of evaluating a new diagnostic assay is by calculation of the kappa value. The kappa value is a measure of the diagnostic agreement between 2 tests beyond the agreement due solely to chance.¹³ Kappa values range from 1 to -1; a kappa value of 1 is interpreted as perfect diagnostic agreement between the 2 tests, -1 means complete disagreement, and 0 means no agreement between the 2 tests except that due to chance. The determination of the kappa value is an appropriate method for comparing a new diagnostic method (MACELISA and PACELISA) with traditional diagnostic methods (EM/IEM). A kappa value of 0.96 for the MACELISA means excellent agreement with results from EM/IEM procedures and demonstrates that MACELISA results can be equally reliable for the detection of BCV antigen in feces. The ability of MACELISA to accurately and reliably detect BCV antigen from WD BCV strains in cell culture and from neonatal calf scours cases suggests that the assay will be useful to epidemiologists and diagnosticians investigating WD and calf BCV infections.

Acknowledgements

We thank Dr. Alejandro Lucchelli and Dr. Ignacio Lanza for their advice and recommendations for the development of both ELISAs. This research was supported by grants from the Ohio Dairy Farmers Federation and from the USDA, Special Animal Health Research Grants program (CSRS-89-34116-4548), and by state and federal funds appropriated to the Ohio Agricultural Research and Development Center (OARDC), the Ohio State University. This report is approved as OARDC article no. 162-94.

Sources and manufacturers

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- d. Titer Tek Multiscan, Flow Laboratories, McLean, VA.
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J Vet Diagn Invest 8: 105-108 (1996)

Experimental infection of swine with a sandfly (*Lutzomyia shannoni*) isolate of vesicular stomatitis virus, New Jersey serotype

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The New Jersey serotype of vesicular stomatitis virus (VSV-NJ) has been the predominant serotype isolated from swine.¹⁸ An enzootic focus of VSV-NJ exists on Ossabaw Island, a barrier island of the Georgia coast, where the virus has been repeatedly isolated from feral swine and the phlebotomine sandfly *Lutzomyia shannoni*,^{2,4,16} a biological vector of the virus. Although infection of Ossabaw Island swine, as determined by a high annual rate of seroconversion, occurs, clinical disease (vesicle formation) is rarely seen.^{6,13-16}

The source of a VSV-NJ isolate may determine its virulence in an animal species. When compared to bovine VSV-NJ isolates, lower titers of swine VSV-NJ isolates are required to produce experimental disease in swine,⁵ the incubation period is shorter, and secondary vesicle formation is more likely to occur.^{5,8} To our knowledge, the virulence of an Ossabaw Island sandfly-derived isolate of VSV-NJ has not been evaluated in swine or other mammalian species.

The intent of this study was 3-fold: 1) to assess the virulence of a 1991 Ossabaw sandfly isolate in swine and to compare the viral dosages used with those viral dosages that could potentially be delivered by a feeding sandfly; 2) to describe the progression of any subclinical or clinical disease; and 3) to determine if viral shedding occurs.

Seven 2-3-month-old crossbreed pigs were used. All pigs were negative for antibodies to VSV-NJ. Three pairs of pigs were inoculated with various dosages of virus, and 1 pig served as a control. Because vesicles on swine on Ossabaw Island have only been observed on the snout,¹⁶ all of the pigs were injected intradermally in the apex of the snout with a single inoculum of 0.1 ml. The viral inocula were prepared from a 1991 Ossabaw Island sandfly isolate that had been passaged once in Vero cells^a and diluted to the appropriate

titer with Dulbecco's phosphate-buffered saline (D-PBS).^b Experimental oral infection of *Lutzomyia shannoni* has resulted in viral titers averaging $10^{4.3}$ plaque-forming units in the heads and $10^{5.3}$ plaque-forming units in the thoraces and abdomens of the flies.³ In an attempt to approximate the viral dose an infected sandfly may be capable of delivering, the middle-dose pair of pigs (C, D) were inoculated with $10^{4.5}$ median tissue culture infective doses (TCID₅₀) of virus. The low-dose pair (A, B) and the high-dose pair (E, F) received $10^{0.7}$ and $10^{7.3}$ TCID₅₀ of virus, respectively. Viral doses were determined via end point titration in Vero cells. The control pig received an injection of D-PBS.

The pigs were restrained daily for physical examination and sample collection. Pigs were examined for the development of lesions, and rectal temperatures were recorded. Blood was collected daily via cranial vena caval puncture for serology, virus isolation, and complete blood counts. Hematologic values were compared with reference values for feeder pigs.⁷ Nasal and tonsillar swabs were collected daily for virus isolation. The pigs were euthanized 10 days postinfection (PI) via intravenous sodium pentobarbital injection, and necropsies were performed. Tissue samples were collected from the snout, right periocular skin, right front leg interdigital skin and coronary band, perineal skin, dorsal lumbar skin, ventral abdominal skin, tonsil, mandibular lymph node, parotid lymph node, retropharyngeal lymph node, nasal mucosa, parotid salivary gland, lung, heart, liver, kidney, spleen, stomach, urinary bladder, and brain. A portion of each sample was placed in transport medium for virus isolation; the remainder of the sample was fixed in 10% buffered formalin for histologic examination.

Tissues and swabs were stored in 1.5 ml viral transport medium consisting of tryptose broth supplemented with antibiotics (1,000 U penicillin G/ml, 1 µg streptomycin/ml, 0.25 mg gentamycin/ml, 0.5 mg kanamycin/ml, 2.5 µg amphotericin B/ml)^b and frozen at -70 C. Pharyngeal and nasal swabs were thawed, vortexed, and centrifuged at 1,500 x g for 10 minutes prior to inoculation on Vero cells. Blood

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Received for publication May 17, 1995.