Assessment of Two Enzyme-Linked Immunosorbent Assay Tests Marketed for Detection of Ruminant Proteins in Finished Feed

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ABSTRACT

The performance characteristics of two enzyme-linked immunosorbent assay (ELISA) test kits, ELISA Technologies’ MELISA-Tek test and Tepnel BioSystems’ BioKit for (Cooked) Species Identification test, designed to detect ruminant proteins in animal feed, were evaluated. The test kits were evaluated by using acceptance criteria developed by the U.S. Food and Drug Administration’s Center for Veterinary Medicine Office of Research for evaluating selectivity, sensitivity, ruggedness, and specificity. The acceptance criteria for determining success used a statistical approach requiring a 90% probability of achieving the correct response within a 95% confidence interval. In practice, this measure requires the test to achieve the correct response 58 times for every 60 samples evaluated, or a 96.7% accuracy rate. A minimum detection level of 0.1% bovine meat and bone meal (BMBM) was required, consistent with the sensitivity of the analytical methods presently used by the U.S. Food and Drug Administration. Selectivity was assessed by testing 60 dairy feed samples that contained no added animal proteins; sensitivity was determined by evaluating 60 samples (per level of fortification) of this same feed that contained 0.025, 0.05, 0.1, 0.25, 0.5, 1, or 2% BMBM. The MELISA-Tek test passed the acceptance set-point criteria for selectivity assessment but failed the sensitivity assessment at all levels except at the 2% level. The MELISA-Tek test came close to passing at the 1% level, detecting true-positive findings at a rate of 93%, but failed at lower levels, in spite of the label claim of 0.5% sensitivity. The BioKit for (Cooked) Species Identification test detected only 2 of 17 samples fortified at the 2% BMBM level and failed to detect any other BMBM-fortified samples. The results of this evaluation indicate that neither test is adequate for regulatory use.

Effective enforcement of the U.S. Food and Drug Administration’s (FDA) 1997 regulation 589.200 (1–3) prohibiting most mammalian proteins in feed for cattle and other ruminants (also known as the “feed ban”) depends in part on effective analytical methods to detect prohibited mammalian proteins. The December 2003 identification of a bovine spongiform encephalopathy–positive cow in Washington State indicated the need for increased surveillance of ruminant feed in the United States. The methods used by the FDA are feed microscopy (6) and PCR (6, 8), where PCR is used as a confirmatory test after a positive finding by feed microscopy. These two methods require dedicated laboratory facilities and trained personnel to conduct the analyses. As such, they are not suitable for manufacturers and end users of animal feed, such as feed mills, ranchers, and farmers. Potential alternatives might be commercial test kits. The FDA evaluated two such tests, the Neogen Reveal Ruminant in Feed test and the FeedChek test of Strategic Diagnostic Inc. (Newark, Del.) (9). Both use lateral-flow technology that uses capillary action and free and bound antibodies to affect detection of the test analyte. Neither test was able to meet the acceptance criteria established by the FDA. However, there were two other tests, ELISA Technologies’ MELISA-Tek (ELISA Technologies, Gainesville, Fla.) and Tepnel BioSystems’ BioKit for (Cooked) Species Identification (Stamford, Conn.), marketed as ruminant-specific and bovine-specific, respectively, that were not evaluated by the FDA during that earlier study.

As with our previous evaluation, little is known about the performance characteristics of these tests beyond the individual manufacturer’s claims. Because of the importance to human and animal health, the Center for Veterinary Medicine decided to evaluate all such test kits marketed for the detection of animal proteins in feed. The goal of this study was to determine whether any of the commercially marketed test kits would provide useful information when used by manufacturers and other end users of animal feed. This report details the results of the evaluation of the two test kits.

MATERIALS AND METHODS

Study design. A statistical approach was used to determine the success or failure of a test to achieve a particular measure. This approach called for a 90% probability of achieving the correct response within a 95% confidence interval. This level of rigor required evaluating 60 feed samples at each level of fortification, or 480 test feed samples per test kit. It further required achieving the correct response a minimum of 58 times for every 60 samples evaluated. Although acceptance criteria for selectivity (truly negative), sensitivity (truly positive), ruggedness, and specificity were...
developed, it was decided that only selectivity and sensitivity would be performed initially. The rationale was that if a test kit could not pass either of these assessments, then there was no reason to conduct the other assessments.

The minimum level of detection that each test needed to achieve to pass the sensitivity portion of the evaluation was a concentration of 0.1% bovine meat and bone meal (BMBM). BMBM was chosen as the test analyte for the sensitivity assessments because this is the animal meal of most interest to the FDA. This level of sensitivity was chosen because first, it is the concentration used in both PCR method validation trials conducted by the FDA (7, 8), and second, it is the level of sensitivity achieved by the feed microscopy method (6). The BMBM used in this study was provided by the Excel Corp. (Wichita, Kans.). It was prepared under conditions reported to reduce bovine spongiform encephalopathy infectivity by 1 log (11), with temperatures during the rendering process ranging from a low of approximately 125°C to a maximum of 131°C (5 min) for a total processing time of approximately 30 min.

The feed samples were placed in Whirl-Pak bags, which were randomly numbered by a random number table. Once all feed samples had been prepared, a second randomization of these samples was performed. This second step was designed to randomly assign the 120 samples for a given level of BMBM fortification to one of the two test kits being evaluated. Once all 480 samples (control and seven levels of BMBM fortification, with 60 samples per level) had been assigned to a given test kit, the complete sets of samples were segregated to prevent inadvertent mixing of the samples. Selectivity assessments were performed by using dairy feed that had not been fortified with any animal proteins. Sensitivity assessments were performed by using dairy feed fortified with BMBM at concentrations of 0.025, 0.05, 0.1, 0.25, 0.5, 1, or 2%. Ruggedness determines the amount of experimental variance that can be tolerated at a given step in the assay and still achieve the correct response. Specificity measures the extent to which the test detects analytes other than those for which the test has been configured. As stated above, the protocol dictated that selectivity and sensitivity assessments be conducted first, followed by ruggedness and specificity evaluations, but only if the test first passed both the selectivity and sensitivity assessments. Only one 15-kg batch of feed was prepared for each level of fortification; this was a sufficiently large batch of feed to supply all the samples needed for both test kits.

The analyses were performed in a blinded fashion; the two analysts conducting the evaluations had no knowledge of the study design or the sample identity. Instrument evaluation of the test results (MELISA-Tek and BioKit) were performed only once on each sample. Visual interpretation of the MELISA-Tek assays were performed by two analysts who independently evaluated each test plate. As a further control measure to maintain their blinded status, all questions, problems, or other issues relative to the conduct of the test were passed to the study director (M.J.M.) through a third party in order to ensure that no hints or clues would be provided to the analysts. The study director and another study participant not involved with performing the evaluation of the test kits prepared the feed samples.

Only one test kit was evaluated at a time. The analysts were given the 480 samples for a given test kit by the study director (M.J.M.), who retrieved these samples upon the conclusion of the selectivity and sensitivity assessments for that test kit. Only after the first set of 480 samples had been analyzed and retrieved by the study director did the analysts begin work on the second test. A single lot of test kits from both manufacturers (ELISA Technologies and Tepnel BioSystems) was obtained in sufficient numbers to perform all the selectivity and sensitivity assessments in order to minimize variability in the test results. However, because we needed to redesign the evaluation of the MELISA-Tek test on the basis of the need to increase the number of test controls on each 96-well plate, additional kits from a different lot were obtained from ELISA Technologies.

This study was conducted according to the FDA's Good Laboratory Practices regulations (12). All phases of this study, including feed preparation and laboratory analyses, were monitored and inspected by the Center for Veterinary Medicine's Office of Research, Quality Assurance Unit. The Quality Assurance Unit reviewed the study protocols and the final reports, as well as all data, data transformations, graphs and tables, statistical analyses, and statements made in the final report and this article.

Test feed preparation. The basal feed used for the true-negative controls and the BMBM fortified feed samples was dairy feed prepared at the Center for Veterinary Medicine's Office of Research feed mill. The feed consisted of corn (30%), oats (35%), soybean meal (26%), dicalcium phosphate (2.5%), dairy premix no. 4 (0.2%; C. S. Akey, Lewisberg, Ohio), soybean oil (1%), dried molasses (2.5%), and salt (NaCl, 1.5%). Fifteen-kilogram batches of dairy concentrate feed containing either no BMBM (control, truly negative), or 0.025, 0.05, 0.1, 0.25, 0.5, 1, or 2% BMBM were prepared. After each batch of BMBM-fortified feed was prepared, the samples (30 to 40 g) were placed into prelabeled Whirl-Pak bags. The control test feed samples were formed into aliquots before preparation of the fortified samples. The individual batches of test feed were prepared beginning with the 0.025% BMBM samples, followed by the 0.05% BMBM feed, with the 2% BMBM fortified feed being prepared last. This approach ensured minimal contamination with an inappropriate amount of BMBM. As a further measure to control contamination, only one batch of feed was prepared at a time. After that batch of feed was prepared and formed into aliquots in the Whirl-Pak bags, the work area and equipment were cleaned before preparing the next batch of BMBM-fortified feed. Feeds containing 0.025, 0.05, 0.1, or 0.25% BMBM were prepared by first mixing the total amount of BMBM for 15 kg of feed with just 1 kg of the basal feed. After mixing the BMBM with 1 kg of feed, this was then added to 14 kg of dairy feed for further mixing to yield the final 15-kg of amount.

Test kit label directions. The feed samples for use with the MELISA-Tek kit were processed and analyzed as detailed in the instructions on the label (5) with the following modifications. Four grams of feed were mixed with 40 ml of extraction solution (supplied in the kit) in a 50-ml conical test tube and allowed to swell for 30 min before heating at 95°C for 15 min. The samples were centrifuged at 10,000 × g for 10 min and the resulting supernatant used in the enzyme-linked immunosorbent assay (ELISA). The label directions call for 5 g of feed to be mixed with 50 ml of extraction solution in a 250-ml flask. Discussion with the company revealed that it is acceptable to scale the assay down, using a 1:10 ratio of feed to extraction solution. Because of the large sample size, we choose to scale down the assay as detailed above. The manufacturer's instructions on how to perform the ELISA portion of the test were followed as written (5).

The test plates were read with a SpectraMax 250 plate reader that was also validated to be compliant with FDA regulation 21, “Electronic Records; Electronic Signatures” (13). After being read on the plate reader, each plate was evaluated visually by two different analysts in accordance with the label directions. The plates were read within 5 min after the instrument evaluation. Their results were recorded on two separate log sheets.
The data from the instrument readings for the MELISA-Tek test were copied into a template that calculated whether a sample was positive or negative, or fell into the retest category. The template also calculated the mean and standard deviation for all samples and controls, and determined whether all the controls were valid on the basis of their standard deviation. All determinations were based on the criteria listed in the kit by the manufacturer. The retest category was developed by the FDA for those test samples that did not meet the criteria of the kit to permit a determination of whether they were positive or negative.

The classifications mentioned above were taken from the MELISA-Tek test kit instructions (5). According to the instructions, an assay may be considered valid if: 1) the mean optical density (OD) of the high positive control (1%) is greater than or equal to 1.000 OD at 450 nm, and 2) if the mean OD of the negative controls is less than 0.150 OD, and 3) if the standard deviation of the control replicates is no more than 0.060 OD. The “and” clauses dictate that all three conditions must be met in order to consider the results of a given plate valid according to the company’s criteria.

The label instructions are silent on whether the third criterion applies to the low-positive control (0.05%). In addition, the example results provided with the kit instructions used only two control samples to calculate mean and standard deviation. A minimum of three samples is needed to calculate standard deviation. Therefore, for this evaluation, all controls, high-positive, low-positive, negative, and plate blanks (which used the extraction solution) were performed in quadruplicate. The controls were performed as two pairs, with each set of pairs placed at different locations on the plate. For the purposes of this study, the low-positive control samples also had to achieve the acceptance criteria.

The kit instructions provided details about how to interpret the test result after instrument reading. Test samples were to be classified as positive if the OD was greater than 0.180 and if the OD was greater than 2 times the mean negative control. Test samples were to be classified as negative if their mean OD was less than 0.180 and if their mean OD was less than 2 times the mean negative control OD. Again, the “and” clauses dictate that all conditions must be met in order to classify a sample as either positive or negative.

Once the data had been classified as either negative, positive, or retest, the raw data were reevaluated by a set-point approach to determine negative and positive samples. Briefly, the results for all samples that fell into the retest category were examined to determine whether the replicate analyses were all above 0.180 OD absorbance units; were all below 0.150 OD absorbance units; or had replicates in which one value was above and one replicate was below 0.180 OD absorbance units. Samples with replicates that were all above 0.180 OD absorbance units were not reanalyzed. Likewise, samples with replicates all below 0.150 OD absorbance units were not reanalyzed. These samples were deemed to be truly positive and truly negative, respectively. Samples in which one replicate was above and the other below 0.180 OD absorbance units were reanalyzed. The reanalyses were performed on a new feed sample withdrawn from the original Whirl-Pak sample bag. On the basis of the second analysis of these samples, a small subset was analyzed for a third time. This latter subset was evaluated by using previously extracted feed samples.

As stated above, the instructions in the MELISA-Tek kit detail how to read the plate visually (manually) (5). The relative colors of the high-positive control, the low-positive control, and the negative control were used as visual reference points. If there was little or no color, the sample was deemed negative. If there was color darker than the low-positive control but less than the color of the low-positive control, the sample was deemed indeterminant. If the color of the sample was darker than the low-positive control, then the sample was deemed positive.

The kit instructions for Tepnel BioSystems’ BioKit for (Cooked) Species Identification test (4) refer to the method of von Holst (11) when analyzing dry feeds. Briefly, 20 ml of water was added to 6 g of dry feed and left for 15 min to swell. One hundred milliliters of normal saline was then added to the mixture, which was mixed and heated to 95 to 100°C for 15 min. The mixture was passed through filter paper, and the resulting supernatant was adjusted to a pH 6.5 to 7.5 (if needed) with small amounts of 2 N HCl or 2 N NaOH, as appropriate.

The test kit is supplied with one positive control (bovine) and three negative control samples (porcine, ovine, poultry). The instructions state that a cutoff value is to be used to determine positive samples; samples with an OD above the cutoff value are deemed positive. The cutoff value is generated by multiplying the F factor with the mean absorbance of the negative controls. For this test, the negative controls are the absorbance values for the porcine and ovine positive control samples. The F factor used by the company for all species is 2.5. The use of this factor is consistent with a detection limit that is at least twice that of the background (i.e., the negative controls). The kit also implies that the test can be read visually. There was no effort made to visually record the results from this test.

**Quality control analysis of feed samples.** While filling the Whirl-Pak bags with feed containing one of the different concentrations of BMBM, samples were collected for PCR analysis to ensure relative homogeneity of the mixing process. Two samples were collected from the top of the mixing chamber immediately before placing samples into the Whirl-Pak bags, two more taken when half of the bags had been filled, with the last two samples were collected after all the Whirl-Pak bags for that level of feed had been filled. These quality control samples were subjected to PCR analysis by means of the method previously validated by the FDA (7), which generates a 271-bp product. PCR analysis demonstrated the presence of BMBM in all fortified feed samples at all levels of fortification. The results also demonstrated that BMBM was present in relatively equivalent amounts from top to bottom, indicating relative homogeneity of the feed within a given level of fortification (data not shown).

**Data analysis.** The data were transferred from the data log sheets into Excel 2002 (Microsoft, Redmond, Calif.). The accuracy of the data entry was determined by an internal data audit, which compared the entries in the Excel spreadsheets with the original data sheets. Any discrepancies in the spreadsheets were then corrected before beginning any statistical analyses. Data were graphed by SigmaPlot version 9.0 (Systat Software, Inc., Point Richmond, Calif.).

**RESULTS**

**MELISA-Tek test kit.** By using the evaluation criteria detailed in the MELISA-Tek test kit to visually assess the results, we found that the kit failed to achieve the acceptance criteria for either selectivity or sensitivity (Figs. 1 and 2, respectively). Although no true-negative samples were scored as positive, analyst 1 scored a large number of true-negative findings as indeterminant (25.8%), meaning that the perceived color was darker than the extract solution blank, but lighter than the color of the low-positive controls. Although analyst 2 scored fewer true-negative find-
ings as indeterminant (7.5%), analyst 2 still scored an unacceptable number of false-positive findings. Furthermore, each analyst independently scored replicates of the same true-negative test sample as negative and indeterminant, respectively.

When visually evaluating the true-positive samples, both analysts scored all the samples ranging from 0.025% through 0.5% as either negative or indeterminant. As the concentration of BMBM increased from 0.025 to 0.5% BMBM, the samples were increasingly classified as indeterminant (Fig. 2). It was only at the 1 and 2% BMBM levels that positive samples were noted. However, at the 1% BMBM level of fortification, only 5 and 16.7% of the samples were identified as positive by analysts 1 and 2, respectively, whereas at the 2% BMBM level of fortification, 51.7 and 71.7% of the samples were identified as truly positive.

When evaluating the MELISA-Tek test by instrument, using a microtiter plate reader and the kit’s acceptance criteria, none of truly negative test samples was recognized as positive. However, only 30% of the samples tested as truly negative, and 70% were classified as requiring retest (Fig. 1). Manual evaluation of these results, focusing solely on whether the samples were below 0.150 OD absorbance units, demonstrated that all the samples classified as requiring retesting were in fact truly negative, indicating that the test is 100% selective (Fig. 2).

All samples at the 2% BMBM fortification level were classified as truly positive (Fig. 3), whereas at the 1% BMBM level, 93% of the samples were detected as truly positive. An accuracy rate below 96.7% will not meet the acceptance criteria, which requires the correct identification of 58 samples out of each 60 samples. However, at the 0.5% BMBM level, only 30% of the samples were classified as
truly positive, whereas at the other lower levels, the rate of
correct classification ranged between 8.3 and 1.7%. Con-
versely, most false-negative responses occurred at the lower
levels of BMBM fortification, starting at a rate of 36.7%
for the samples containing 0.025% BMBM and declining
to a false-negative rate of 3.3% in samples containing 0.5%
BMBM. The remaining samples were classified as requiring
retesting. However, this level of accuracy is possible only
if one does not use the manufacturer’s criteria for deter-
mining whether the data on a particular ELISA plate are
valid. As stated in the instructions, the data on a plate are
considered valid if, in addition to the other two conditions,
“the standard deviation of the control replicates is no more
than 0.060 O.D.” (5). Ten of 15 plates failed to meet this
third criterion; on one plate, the negative control samples
failed this criterion, and on nine plates, the low-positive
control failed to meet this criterion.

The instrument-generated data were reviewed to deter-
mine how many of the retest samples were above the 0.180
OD absorbance units or below 0.150 OD absorbance units,
and to determine how many samples had replicate values
that widely varied from each other, focusing mainly on
those samples in which one replicate was above 0.180 and
the other replicate was below 0.180 OD absorbance units.
The latter was an arbitrary assessment; these variations had
to potentially affect a determination of whether a sample
was positive or negative for BMBM, whereas samples with
a large variance but with both replicates at or above 0.180
OD were still going to be classified as positive. The detailed
analysis of the samples in the retest classification demon-
strated that most these samples were truly negative because
both replicates were well below the 0.150 OD value (Fig.
3). The reason for the retest classification of this subset of
samples was that their mean OD was not less than two
times the negative control, one of the two criteria needed
to classify a sample as negative according to the kit label
instructions.

Thirty-nine samples were selected for reanalysis by us-
ing a new feed aliquot from those samples because one of
the replicate values was above 0.180 OD and the other was
below 0.180 OD. Out of these samples, a small subset of
10 samples still had replicate analyses that widely varied
from each other. Those samples were analyzed for a third
time; however, this time, the samples were analyzed by us-
using the material previously extracted for the first reanalysis.
There were still a few samples exhibiting poor replication,
as discussed below. The reanalysis of these samples and
re-examination of the data by a set-point analysis demonstrat-
ed that most of the samples previously classified as requir-
ing retesting were in fact truly negative (Fig. 4). Thus, only
at the 2% BMBM level does the test meet the acceptance
criteria; it is close at the 1% BMBM level; and it rapidly
falls below the 0.5% BMBM level, contrary to the claims
on the label of a 0.5% level of sensitivity.

The issue of sample replication was examined in two
ways, one deliberate and one accidentally. During the last
reanalysis of the test samples, three samples previously
shown to be positive with relatively small variances were
reexamined. Four replicates for each sample were examined
within a single column (i.e., on one strip), and two sets of
four replicates were examined on different columns (i.e.,
on different strips). These results demonstrated problems
with obtaining results with minimal variance, both within
a single strip of wells (same column) and between wells on
different strips (same row, different columns). The varian-
ces with a single column for these three samples were 0.047,
0.569, and 0.070 OD absorbance units, respectively. Their
previous variances were 0.003, 0.093, and 0.055 OD ab-
sorbance units, respectively. The variances between the
replicates in different test strips were even greater than the
within-strip variation, with differences of 0.105, 0.364, and
0.327 OD absorbance units, respectively, for these three
samples.

One possible explanation for this problem might be in
the manufacturing of the plastic eight-well test strips used
in this ELISA. Therefore, one complete ELISA plate (12 eight-well strips) was optically analyzed before being used in the analysis of test samples. Analysis of this blank ELISA plate demonstrated no optical differences between any of the 96 wells. Subsequently, when this same ELISA plate was used to analyze test samples, two complete eight-well strips along with two rows of five wells each were not needed for analysis of test samples. Nothing was added to these 24 wells. These wells were merely processed along with the rest of the plate for the ELISA portion of this test. Unexpectedly, 8 of 24 wells were truly negative—that is, they only contained the ELISA test reagents that demonstrated significant absorbance values. Two wells had OD values consistent with the criterion for classifying a sample as positive—that is, their absorbance values were greater than 0.180 OD absorbance units. The other six wells had OD values sufficient to cause true-negative samples to be potentially classified as positive samples, because many of the true-negative test samples by themselves had values at or slightly above 0.040 OD absorbance units.

**BioKit for (Cooked) Species Identification test kit.**

The initial work that used this test involved either pure BMBM samples or feed samples with and without 2% BMBM. The results of these analyses demonstrated that the kit could barely detect pure BMBM samples (data not shown); however, it could consistently yield positive results for all BMBM samples. A total of 126 test feed samples of 480 were evaluated with this kit. Only two samples tested positive for bovine protein; both were in the 2% BMBM fortification group (Table 1). This represents a detection rate of 11.8% (2 of 17). Because the acceptance criteria called for a detection rate of at least 58 of 60, the test had already failed to meet the acceptance criteria. No amount of additional sample testing (of the remaining 354 samples) would change this fact. Therefore, a decision was made to stop further work on this test kit.

**DISCUSSION**

Neither ELISA Technologies’ MELISA-Tek test nor Tepnel BioSystems’ BioKit for (Cooked) Species Identification test met the acceptance criteria for both selectivity and sensitivity. The MELISA-Tek test failed to achieve the desired selectivity when the plates were analyzed by using a microtiter plate reader and evaluating the data according to the kit instructions, which indicated that the reason for this failure may be inappropriate guidance on interpretation of the test results. The true-negative samples typically had low absorbance values that were usually close to the values for the negative controls. This fact alone made it impossible for many of the true-negative feed samples to be classified as negative; their mean OD was not less than twice the mean OD of the negative controls. The more important criterion for determining true-negative and true-positive findings for this test appeared to be an OD value below 0.150 or above 0.180, respectively. Therefore, a study-specific criterion was applied to the evaluation of test results—namely, whether the samples were below 0.150 OD absorbance units (for true-negative findings) or above 0.180 OD absorbance units (for true-positive findings). It was only after applying this study specific criterion (a set-point approach) that all the truly negative samples were correctly designated.

The MELISA-Tek test claims a 0.5% detection level of ruminant meat and bone meal in a grain-based feed sample. However, it also claims a 0.05% detection level of ruminant BMBM in grains on the basis of results that use a 1:10 dilution of the original sample. The BioKit test does not make specific detection level claims. Rather, the instructions state, “It has been reported that the kits can determine species content in feeds containing rendered animal meat and bone meal.... Although BioKits has performed an amount of animal feed testing but it [Tepnel BioSystems] has not been able to obtain sufficient quantities of MBM sample to adequately validate this aspect of the test’s performance” (4).

The MELISA-Tek test could not achieve the claimed 0.50% sensitivity, regardless of whether data interpretation used the instructions from the kit or a set-point approach. This test could only meet the acceptance criteria for the 2% level, achieving a success rate of 100%. The kit came close at the 1% BMBM level, achieving a 93% success rate in identifying true-positive findings. As stated previously, to achieve the acceptance criteria with just 60 samples requires correctly identifying at least 58 of those test samples, which in practice equals a 96.7% accuracy rate. The kit could not achieve the stated claim of the kit of 0.5% BMBM. As stated above, this level of accuracy at the 2% BMBM level is possible only if one ignores the manufacturer’s criteria for determining whether the data on a particular ELISA plate are valid. Because most of the issues with determining plate validity were due to problems with the low-positive control, clear directions stating whether this control is or is not needed to assess plate validity could potentially eliminate this problem. The issue of the one

**TABLE 1. Results for sensitivity and selectivity assessment of Tepnel’s BioKit for (Cooked) Species Identification assay**

<table>
<thead>
<tr>
<th>Detection level for bovine meat and bone meal (%)</th>
<th>No. correct/total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16/16 (100)</td>
</tr>
<tr>
<td>2</td>
<td>2/17 (11.8)</td>
</tr>
<tr>
<td>1</td>
<td>0/17 (0)</td>
</tr>
<tr>
<td>0.5</td>
<td>0/18 (0)</td>
</tr>
<tr>
<td>0.25</td>
<td>0/15 (0)</td>
</tr>
<tr>
<td>0.1</td>
<td>0/14 (0)</td>
</tr>
<tr>
<td>0.05</td>
<td>0/13 (0)</td>
</tr>
<tr>
<td>0.025</td>
<td>0/16 (0)</td>
</tr>
</tbody>
</table>

* Only the first 116 samples were analyzed. Because not all 60 samples per fortification level were analyzed, there would be no expectation that the same number of samples in each group would have been analyzed at this point, because these sample numbers had been randomly assigned to a given fortification level.

Although there were no false-positive findings detected with this limited sample set, there can be no expectation that this is a valid result because no true-positive results could be detected.
plate that failed this assessment as a result of variable results with the negative control could be addressed by the inclusion of directions requiring the controls to be performed in triplicate (as needed to determine standard deviation), and OD values less than zero should be considered as zero.

The issues with sample replication with the MELISA-Tek test after analysis with a plate reader suggest that there are problems with how the assay has been designed as opposed to problems with the optical quality of the individual eight-well test strips used with this kit. Because there were no differences in the optical quality of the test strips when the empty plate was read—that is, before the plate is used for an assay—the optical clarity of these strips is not an issue. However, these same wells exhibited marked OD levels at the conclusion of the ELISA process when the only thing added to these wells were the individual ELISA reagents: antibody, substrate, and wash reagent. A possible reason for this outcome is an inadequate amount of blocking agent applied to the wells at the point of kit manufacture. It is well known that incomplete blockage of the surface of the individual wells will result in nonspecific antibody binding, causing a false-positive signal to be produced.

The results for the MELISA-Tek kit failed to achieve the performance goals for both selectivity and sensitivity when evaluated visually. This failure is not due to differences in how the plates were interpreted by the two analysts. There was very good agreement between the two analysts evaluating the test plates when assessing sensitivity (true-positive samples). There was poor agreement in the interpretation of the test results when selectivity assessments (true-negative samples) were being determined.

The Tepnel BioSystems BioKit for (Cooked) Species Identification test detected only 2 of 17 samples fortified at the 2% BMM level, and failed to detect any other BMM-fortified samples (Table 1). The inability of the test could have been predicted on the basis of pretstudy work that used pure BMM. The results of these analyses demonstrated that 100% BMM yielded measurable signals that were on average 0.027 to 0.185 OD absorbance units above background. Clearly, this response range was insufficient to yield a detectable signal when the sample analyte was diluted 1,000-fold in the test feed matrix.

The failure of the BioKit test is all the more perplexing when one considers the fact that both test kits use the same antibodies (10). However, the BioKit instructions state that (regarding detection of meat and bone meal [MBM]) “it [Tepnel BioSystems] has not been able to obtain sufficient quantities of MBM samples to adequately validate this aspect of the test’s performance” (4). In the same section, the instructions also state that it is up to users to determine whether a given batch of test samples is adequate for their needs.

The instructions for both kits were at times confusing and difficult to read. Critical information on how to conduct each assay was buried in the kit instructions. For example, in the instructions for the Tepnel kit, the directive to use the porcine and ovine standards as the negative controls for detection of bovine protein is found on page 11, well after the directions on how to conduct the test (4). Although instructions that were clearer and easier to follow would have been helpful when performing the test, they would not have affected the outcome of this study.

The failure of these two ELISA-based assays to attain the acceptance criteria suggests that the failure of these two tests, along with two lateral-flow tests (Neogen’s Reveal for Ruminant in Feed test and Strategic Diagnostic’s FeedChek test) that had been previously evaluated (9) does not appear to be a failure of a given technology, but rather may represent common factors. The two lateral-flow strip tests used different antibodies, whereas the two ELISA tests used the same antibody. Indeed, only Strategic Diagnostic Inc.’s FeedChek test uses a unique antibody; the other three test kits all use the same antibodies. The Reveal, MELISA-Tek, and BioKit tests all suffered from a lack of sensitivity, at least as required for the FDA’s evaluation of these test kits. In contrast, the FeedChek test, which uses different antibodies, suffered from a lack of selectivity; it had an unacceptable level of false-positive findings. These observations suggest that one reason for failure of these four tests to attain the acceptance criteria may be with the antibodies used for these tests. Whether this represents a failure to use appropriate antibody levels or is an issue with the antibodies themselves cannot be assessed at this time. In conclusion, these two ELISA tests failed our evaluation criteria, thus diminishing their usefulness to serve in a regulatory capacity.

REFERENCES


5. MELISA-Tek kit instructions. ELISA Technologies, Gainesville, Fla.


