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Research Note

Evaluation of an Enzyme Immunoassay for the Detection of Central Nervous System Tissue Contamination at the Slaughterhouse

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ABSTRACT

To protect public health from bovine spongiform encephalopathy, European Commission Regulation EC 1139/2003 on monitoring programs and specified risk material requires that as of 1 October 2003, each member state has in place a sampling plan with an appropriate laboratory test to detect central nervous system (CNS) tissue in bovine head meat harvested at slaughterhouses or cutting plants. With this study, we wanted to evaluate the accuracy and reliability of an enzyme immunoassay, the RIDASCREEN Risk Material 10/5, in targeting a CNS-specific marker, the glial fibrillary acidic protein. A receiver operating characteristics curve was plotted to identify the best cutoff of CNS concentration. Reliability was calculated by Cohen's kappa on data from two diagnostic sessions. Test performance showed high sensitivity and specificity (97.9 and 97.4%, respectively) for a cutoff value between positive and negative at a CNS concentration of 0.049%; reliability of test precision was also very good. When these criteria are applied, the RIDASCREEN Risk Material 10/5 test appears to be a reliable tool for monitoring CNS tissue contamination in meat. This diagnostic procedure should therefore be recommended for national application in monitoring programs.

The bovine spongiform encephalopathy (BSE) epidemic in Europe and the emergence of variant Creutzfeldt-Jacob disease in humans of probable dietary origin *(11),* i.e., humans consuming food containing bovine-derived materials infected with BSE, has led the European Community to progressively strengthen rules to prevent contamination of the food chain with the BSE agent. Of the measures the European Commission has adopted, EC Regulation 1139/2003 *(3)* requires that as of 1 October 2003, member states have diagnostic systems in place for the detection of central nervous system (CNS) tissue contamination in head meat from bovine animals at slaughterhouses and cutting plants. Owing to the early accumulation of the BSE agent in the CNS *(9),* the specified risk material ban is considered the main preventive measure against human transmissible spongiform encephalopathy (TSE) exposure.

To verify the presence of CNS tissue contamination in final meat products but not in raw materials, a number of methods have been developed that can detect specific CNS markers. Of these, the neuron-specific enolase *(5)* and glial fibrillary acidic protein (GFAP) *(7, 10)* are based on immunological methods, whereas cholesterol *(5)* and fatty acids *(6)* detection methods are based on enzymatic and chromatographic–mass spectrometric techniques, respectively. More recently, a reverse transcription PCR to detect GFAP mRNA has been investigated *(8).* However, an evaluation of test accuracy on a significantly large number of reference samples has never been carried out. In 2004, the Italian Ministry of Health requested the Italian National Reference Center for TSE evaluate which of the two techniques the EC Food Production Unit of the Joint Research Center evaluated in 2002 *(1)* could be suitable for monitoring CNS contamination of meat at slaughtering plants. In one method, neuron-specific enolase is detected by gel electrophoresis and Western blot analyses; the other method involves use of an enzyme-linked immunosorbent assay (ELISA) technique for determining the presence of GFAP *(5, 7).*

The GFAP-detection test kit, the RIDASCREEN Risk Material 10/05 (RRM 10/05; R-Biopharm GmbH, Darmstadt, Germany), was offered free of charge by the vendor for comparative evaluation.

RRM 10/5 is the latest version of an enzyme immunoassay for the quantitative analysis of GFAP, wherein sampling is done by swabbing a representative area of meat surface to detect levels of CNS contamination (Fig. 1).

With this study, we wanted to evaluate the test's accuracy and reliability in detecting the presence of CNS tissue in raw cattle meat and whether it could be suitably applied in national monitoring programs.

MATERIALS AND METHODS

CNS-negative meat. The material was obtained from the hind legs of two fallen stock bovines (least likely to be contam-

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FIGURE 1. *Sampling at the slaughterhouse. Swabbing of an area 10 by 10 cm of the surface of dissected head muscles after decapitation.*

inated by CNS tissue). The material (about 8 kg) was cleaned by rinsing in distilled water, cut into small cubes, homogenized (cycles of 10,000 rpm for 18 s) in a blender (Grindomix GM 200, Retsch, Haan, Germany), and stored at −20°C in aliquots of about 200 g immediately after processing.

CNS-negative and -positive meat samples. Five hundred three true negative samples were prepared with aliquots of the homogenized meat, one for each day of analysis. A total of 558 true positive samples were prepared by mixing bovine brain with the homogenized meat aliquots to produce specimens at three brain concentrations (0.1, 0.2, and 0.4% brain tissue) in the mixtures. A 0.1% (wt/wt) mix of bovine brain and muscle was prepared by mixing 0.2 g of bovine brain homogenate with 199.8 g of homogenized meat, followed by processing of the mixture in a blender (Grindomix GM 200, Retsch) at 10,000 rpm for about 40 s in 10-s intervals. The next mix in this series (0.2% brain in meat) was prepared by mixing 199.6 g of meat with 0.4 g of bovine brain, and then homogenizing it as described above. A third mix of the series (0.4% brain in meat) was prepared in a similar manner.

All samples were presented for testing in a coded format.

RRM 10/5. Sample processing and analyses were carried out according to the standard operating procedure based on the manufacturer's instructions. In brief, one sampling swab was inserted several times in each meat sample, and then squeezed into a test tube containing 1 ml of diluted sample dilution buffer.

In our study, each standard solution provided by the kit was added in duplicate (0, 0.1, 0.2, and 0.4% CNS concentration), and 50 μl of 24 prepared meat standards was performed each time. The standards and the samples were added to microtiter strip wells coated with the anti-GFAP primary antibody. Next, 50 μl of anti-GFAP/peroxidase conjugate was added to the bottom of the wells, mixed, and incubated for 10 min at room temperature (20 to 25°C) in the dark. After three washing steps, 100 μl of substrate/chromogen was added to each well, mixed, and incubated for 5 min at room temperature in the dark. The reaction was stopped by adding 100 μl of stop solution. Absorbance at 450 nm (Tecan SUNRISE, Gröder, Salzburg, Austria) was measured within 15 min after adding the stop reagent. The RIDA SOFT Win software (R-Biopharm AG) allows automated data retrieval from the reader and calculation of results by linear regression (Fig. 2). Total test time was about 20 min.

Sensitivity and specificity calculation and cutoff selection. All data were recorded on a datasheet (Microsoft Excel) and in an ad hoc dataset for statistical analysis with the Stata 8.2 software package. The results (CNS concentration expressed in percentages) were expressed as continuous values; however, to identify the best cutoff discriminating positive and negative samples, the results were grouped in classes. Because of their uneven distribution, with most of them at very low concentration values, a convenience criterion was used to create 17 classes: 10 classes were obtained between 0.01 and 0.1 (pace 0.01) and an additional 7 classes between 0.1 and 0.8 (pace 0.1). Based on these classes, receiver operating characteristics (ROC) curves for parametric data were plotted to discriminate between two mutually exclusive states (positive or negative) of the samples. Using the prescribed

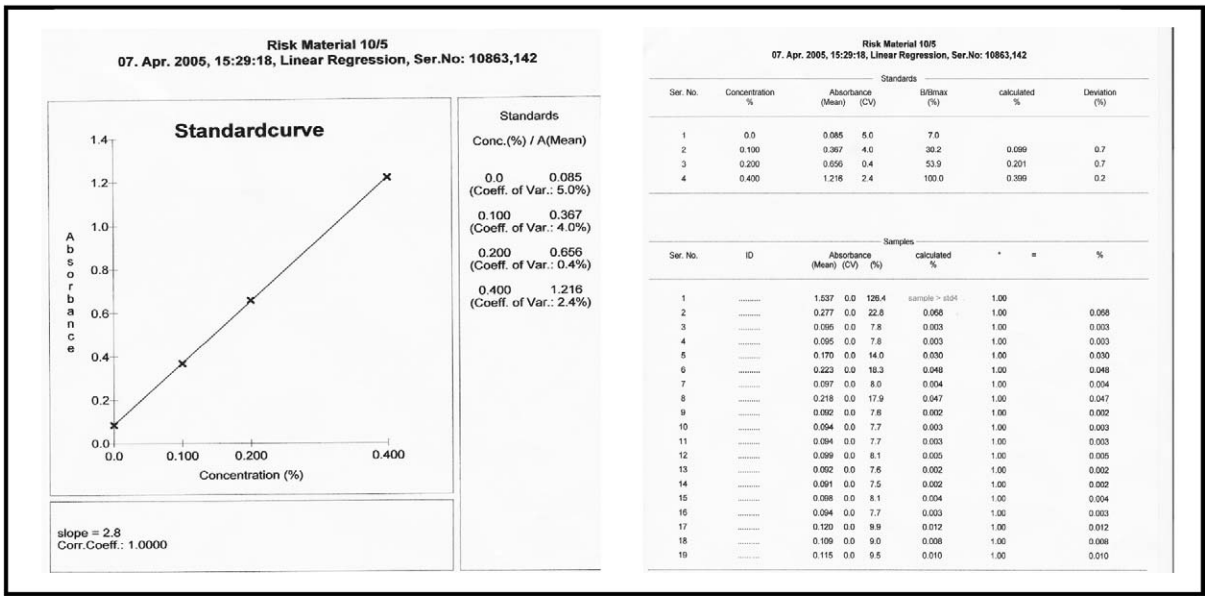

FIGURE 2. *Software RIDA SOFT Win of R-Biopharm. On the left, the absorption values of the standards are plotted against the risk material contents in percentages; on the right, results of linear regression analysis and calculation are shown.*

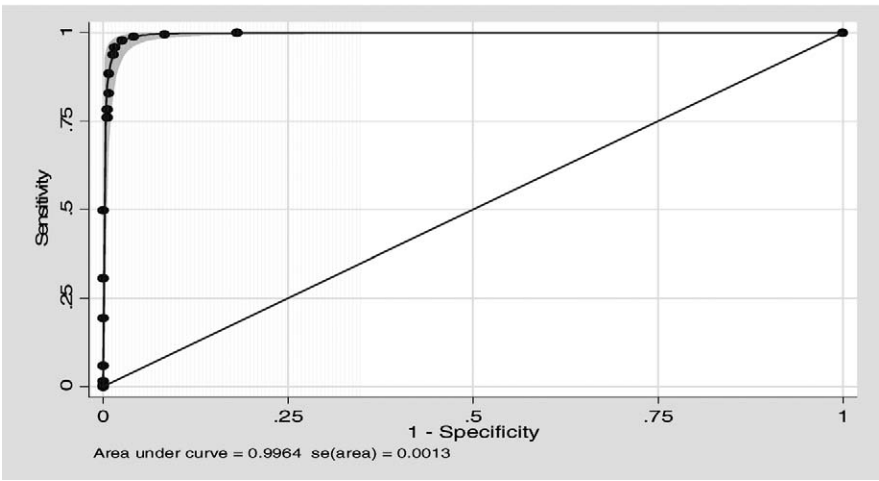

FIGURE 3. *The ROC curve for sensitivity, specificity, and cutoff selection.*

threshold values, the test was considered qualitative in order to evaluate its reliability.

Reliability. To evaluate the reliability of the method in terms of interrater and intrarater agreement, a subset of 32 samples (2 positive samples and 30 negative ones) was analyzed by two different observers (operator 1 and operator 2) who repeated the analysis on two different consecutive days. Cohen's kappa and 95% confidence intervals [CIs] were calculated comparing separately (i) the results obtained by operator 1 on day 1 with those obtained on day 2 (intrarater), (ii) the results obtained by operator 2 on day 1 with those obtained on day 2 (intrarater), (iii) the results obtained by operator 1 with those by operator 2 on day 1 (interrater), and (iv) the results obtained by operator 1 with those by operator 2 on day 2 (interrater).

RESULTS

Figure 3 shows the results of cutoff calculation. The ROC curve area was 0.996 (95% CI: 0.993 to 0.999), indicating high sensitivity and specificity. The graph coordinates represent sensitivity and rate of false positives. Coinciding at the point of maximum sensitivity value and minimum rate of false positives, the cutoff value was fixed at 0.049, which corresponds to a CNS concentration of 0.049%. In this case, the cutoff guarantees the highest level of sensitivity (97.9%; 95% CI: 0.89 to 1.00) and specificity (97.4%; 95% CI: 0.89 to 1.00).

The results of the assessment of interrater and intrarater agreement are reported in Table 1.

TABLE 1. *Cohen's kappa (κ) and 95% confidence interval (CI) calculated by comparing the results obtained by two different observers (operator 1 and operator 2) who repeated the analysis on two different consecutive days (n = 32, with 2 positive and 30 negative samples)*

Interrater reliability	
Results of operator 1 vs operator 2 on days 1 and 2	Results of day 1 vs day 2 separately for the two operators
Day 1, κ = 1.00 (95% CI: 0.65–1.00)	Operator 1: κ = 0.78 (95% CI: 0.45–1.00)
Day 2: κ = 0.78 (95% CI: 0.45–1.00)	Operator 2: κ = 1.00 (95% CI: 0.65–1.00)

DISCUSSION

EC Regulation 1139/2003 provides indications for monitoring CNS contamination in head meat of bovine animals older than 12 months, but does not specify the type of test to verify contamination or the accepted CNS concentration threshold. We evaluated the performance of an ELISA system for the quantitative detection of GFAP, a cell marker highly concentrated in CNS, and fixed a threshold at the highest sensitivity value and the lowest rate of false positives.

With this approach, the test can be considered qualitative, because it excludes the variation dependent on GFAP positivity related to the level of peripheral nerves and not to CNS tissue present in meat tissue samples *(7).*

ROC curves draw an area under the curve that is a global summary statistic of diagnostic accuracy, wherein the larger the area, the higher the sensitivity and specificity, as there is no overlap of values of the two groups (positives and negatives) *(4).* The obtained area was close to 1, indicating that our data showed very high levels of qualitative accuracy.

The evaluation of test precision showed an excellent agreement between analytical sessions; the large CIs obtained can be related to the small sample size used for the assessment ($n = 32$). From this we can conclude that RRM 10/5 may be a simple valid diagnostic test for routine monitoring of CNS tissue presence in meat at the slaughterhouse.

A major limitation to the technique is that it cannot discriminate between CNS tissue from animals of different ages. This constitutes an important drawback, because the current legal definition of specified risk material includes only bovine animals older than 12 months *(2).* The problem may be overcome, however, if animals subjected to CNS removal (>12 months) and younger ones are slaughtered in separate sessions. Provided that cleaning and disinfection are carried out between the two sessions, an indication of specified risk material-specific contamination can be obtained.

The safe removal of CNS tissue at the slaughterhouse depends mainly on how carefully the heads are handled and

on safe sealing of the frontal shot hole and the foramen magnum. However, head meat is often harvested from heads with damaged eyes or which have not been properly sealed. Data obtained from a national sampling plan applying the above criteria would give a reliable indication for proper head harvesting. Depending on the number of positive samples detected, contamination could be reduced by implementing such various measures as providing operator working instructions, use of impermeable and durable stoppers, and exclusion of heads with damaged eyes from harvesting.

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