Agar gel immunodiffusion test for the detection of bovine leukemia virus antibodies: lack of trans-Atlantic standardization

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Abstract

Two agar gel immunodiffusion (AGID) kits for the serodiagnosis of bovine leukemia virus (BLV) were imported from Europe and were compared with North American kits. The BLV AGID kits from North America and from Europe differed significantly. The punches were different, as were the pattern distribution in the agar of the reference and the test sera, resulting in differences in the reading of the immunoprecipitation lines. Based on the testing of 1200 serum samples from cattle, the European kits gave a good correlation with the American kits, as indicated by their respective kappa values. However, the European kits were found to be less sensitive when evaluated against weakly positive samples from field specimens or following a dilution trial. Only 65% and 50% of the weakly positive samples detected by the American kit #1 were detected by the European kits #2 and #3, respectively. The American kit was also capable of detecting BLV antibodies in 45% of strongly positive samples diluted 1/50 in negative sera, while antibodies were detected in only 15% of the samples with the European kit #2 and in none of the samples with the European kit #3. False negatives were also detected with the European kits. Among the false negatives, the degree of expected reactions was weak (European kit #2) or of varying degrees of positivity (European kit #3). Besides the differences in format and performance, the BLV-AGID kits in Europe are evaluated with the National Standard Serum E4 while a proficiency panel composed of a quadruplicate set of 10 reference sera is used in Canada to monitor the kits. Based on the overall observations, we noted a lack of standardization between the BLV-AGID kits used in North America and in Europe.

Résumé

Deux trousses d'immunodiffusion en gel d'agar (IDG) utilisées pour le diagnostic de la leucose bovine ont été importées d'Europe et comparées avec des trousses nord-américaines. Les trousses nord-américaines et européennes présentaient des différences significatives. Les poinçons utilisés lors des épreuves étaient différents, de même que la distribution des sérums de référence et de ceux à tester, entraînant une différence dans la lecture des lignes d'immunoprécipitation. Les trousses européennes étaient comparables aux trousses nord-américaines selon leur valeurs kappa respectives obtenues par l'analyse de 1200 échantillons. Cependant, les trousses européennes étaient moins sensibles pour déceler les échantillons faiblement positifs provenant d'échantillons diagnostiques ou d'une expérience de dilution. Seulement 65 % et 50 % des échantillons faiblement positifs détectés par la trousse américaine #1 furent détectés par les trousses européennes #2 et #3 respectivement. La trousse américaine était de plus capable de détecter des anticorps anti-leucose bovine dans 45 % des échantillons fortement positifs dilués 1/50 avec du sérum négatif de bovin, alors que seulement 15 % (trousse #2) et 0 % (trousse #3) de ces échantillons étaient décelés par les trousses européennes. Des échantillons faux négatifs furent également détectés par les trousses européennes. Parmi les réactions faussement négatives, les réactions anticipées était faiblement positives (trousse #2) ou de différents degrés de positivité (trousse #3). À part les différences de format et de rendement, les trousses européennes d'IDG sont évaluées avec le Standard National E4 alors qu'un quadruplicat de dix sérums de référence composant une épreuve de compétence standardisée sert à l'évaluation des trousses utilisées au Canada. Ces observations dénotent une différence entre les standards nord américains et européens pour les trousses d'IDG de la leucose bovine.

(Traduit par les auteurs)

Introduction

Enzootic bovine leukosis (EBL) is a retroviral disease of cattle characterized by the development in older animals of B-cell lymphosarcomas or localized lymphoid tumors. Most of the animals infected with the bovine leukemia virus (BLV) exhibit a persistent B-cell lymphocytosis or remain subclinical (1,2). Although EBL disease is found in less than 5% of the BLV- infected animals (3,4), BLV infection is widespread in Canada and the United States (5,6).

The agar gel immunodiffusion (AGID) test replaced the Bendixen key soon after the serological test was developed in the mid-1970s (7–9). The AGID test is simple and easy to perform and has been

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Received August 27, 1999.

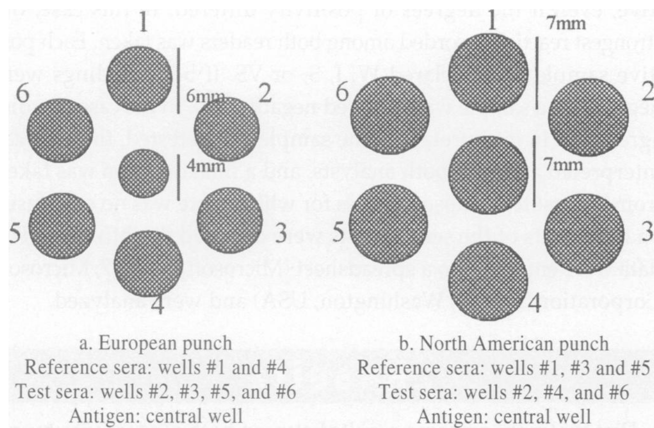

Figure 1. Characteristics of the European and the North American punches used for the BLV AGID test.

adopted as a standard test method by the Office International des Épizooties (OIE). This test is requested by many countries as a prerequisite for the importation of cattle from Canada. Traditionally, the AGID kits approved for the official testing by Agriculture and Agri-Food Canada, and more recently by the Canadian Food Inspection Agency (CFIA), are manufactured either in Canada or in the United States. These kits are validated by the CFIA to ensure performance and quality. To be valid, a commercial kit must successfully pass a proficiency panel composed of a quadruplicate set of 10 reference sera. The BLV-AGID kits are also manufactured in other countries, notably in western Europe. This study was done to compare North American to European AGID kits used for the serodiagnosis of bovine leukosis.

Materials and methods

Commercial BLV-AGID kits

Two North American and 2 European AGID kits were compared. The BLV-AGID (kit #1) was manufactured by Rhone Merieux Inc, Athens, Georgia, USA. This kit had been officially approved by the Canadian Food inspection Agency and was used as the reference standard kit in this study. The European BLV-AGID (kit #2) was obtained from Dr. A.G. Bommeli, Bern, Switzerland. The European BLV-AGID (kit #3) was obtained from Rhône Mérieux Diagnostics, Lyon, France. Most of the positive samples used in the validation study ($n = 415$) were previously tested by a Canadian BLV-AGID (kit #4) produced by the Institut Armand-Frappier, Québec. Kit #4 was also officially approved by the CFIA for the testing of BLV in Canada; however, at the time of the study, a limited number of kits were available. All the kits from each manufacturer were from a single serial lot number.

For the European kits, the product information insert provided by each manufacturer was strictly followed. Both European kits provided a mixture of agar, a positive control (reference) serum, and the bovine leukosis antigen for the AGID test. Kit #3 also provided the diluents. To perform the test according to the manufacturers' protocols, the required BLV-AGID punch in use in Europe was obtained. This punch consisted of a central well 4 mm in diameter and 6 surrounding wells 6 mm in diameter, all 3 mm apart. The wells were filled according to the specifications of each kit insert. The central well received 30 μL (kit #2) and 32 μL (kit #3) of the dissolved antigen. The surrounding wells 1 and 4 received 60 μL (kit #2) and 73 μL (kit #3) of the reference serum and wells #2, 3, 5, and 6 received the same amount of the serum sample to be tested (Figure 1a).

For the North American kits, the CFIA BLV official protocol, based on the information provided by the manufacturers, was followed (10). Both kits provided the BLV antigen, reference serum, negative and weak positive controls. The agar was prepared according to the official protocol. A recommended punch was used. The pattern of the punch consisted of a central well and 6 surrounding wells, all 7 mm in diameter and 3.0 mm apart. All wells were filled with 80 μL of their required sample. The central well was filled with the antigen, wells #1, 3, and 5 were filled with the reference serum, and wells #2, 4, and 6 were filled with the serum sample to be tested (Figure 1b).

Samples

A total of 1200 serum samples from cattle older than 6 mo of age were used for the comparative study. These samples were randomly selected from a bank of samples collected at the CFIA Retrovirology Centre of Expertise. They were collected from artificial insemination centres and from animals tested for disease control and export purposes. The samples were tested with the American BLV-AGID test kit (kit #1) and/or the Canadian BLV-AGID test kit (kit #4) prior to being banked. Based on these test results, a total of 704 BLV-AGID-negative sera, 490 BLV-AGID-positive sera, and 6 doubtful samples were selected and mixed in storage boxes. For the dilution trial study, 10 negative and 40 strongly positive samples were selected from the bank. They were diluted from neat to 1/50 using the same batch of BLV-negative bovine serum. Each sample was aliquoted in replicates and coded so that the analysts were not aware of the samples' identities while performing and interpreting the tests. Aliquoted samples were frozen at $-20°C \pm 5°C$ until use. For each kit in the validation study, a vial of each series was thawed, the serum was resuspended and the test was performed by BLV-AGID analysts certified by the CFIA, according to the manufacturer's instructions and/or the official protocol (10). The test reactions were interpreted in a dark room over a beam source of light; this was done independently by 2 certified analysts, each using individual daysheets, so that each was unaware of the other's results. Data were compiled at the end of the day. The samples for which a disagreement occurred between both readers were retested by the same analysts, using another vial from the same series.

Test reactions

The pattern of disposition of the reference and the test sample wells in the European, as compared with the North American kits, were different. There was only one reference line for each test sample in the European kits, whereas the North American kits had 2 lines. A sample was declared positive if it formed a precipitation curve specific to the viral antigen and if this curve formed a line of identity with the reference serum. It was declared negative if it did not formed a specific line with the antigen and if it did not bend the line of the reference serum. The degree of positivity was

Table I. Comparison of the American BLV-AGID kit #1 with the European BLV-AGID kit #2 on 1200 serum samples

American	European BLV-AGID kit #2			
BLV-AGID kit #1	Positive	Negative	Doubtful	Total
Positive	483	7	0	490
Negative	0	701	3	704
Doubtful	0	6	0	6
Total	483	714	3	1200

Table II. Comparison of the American BLV-AGID kit #1 with the European BLV-AGID kit #3 on 1200 serum samples

American	European BLV-AGID kit #3			
BLV-AGID kit #1	Positive	Negative	Doubtful	Total
Positive	471	12	7	490
Negative	11	687	6	704
Doubtful	1	5	0	6
Total	483	704	13	1200

determined according to the following criteria: 1) Weakly positive (W) — The end(s) of the reference line(s) curved toward the antigen well but did not form a complete line between the antigen and test serum well; 2) Intermediately positive (I) — The end(s) of the reference line(s) curved toward the antigen well and formed a complete line between the antigen and test serum well. This line just touched the test serum well or was located very close to it. It was or was not equal in width and sharpness to the reference line; 3) Strongly positive (S) — The end(s) of the reference line(s) curved toward the antigen well and formed a complete line between the antigen and test serum well. This line was located at or approximately halfway between the antigen and test serum well. The precipitation line was as sharp and bright as the reference line; 4) Very strongly positive (VS) — The end(s) of the reference line(s) curved toward the antigen well and formed a complete line between the antigen and test serum well. This line was closer to the antigen well than the test serum well. It was a "diffused line" that was not as sharp or bright as the reference line; sometimes there was no immunoprecipitation line between the antigen and test serum wells.

The W, I, S and VS reactions corresponded respectively to the curved line (CL), weak identity line (WIL), identity line (IL), and diffused line (DL) of the CFIA protocol (10). Reactions that were neither clearly negative nor clearly positive were declared doubtful (D). For example, a sample was declared D if the end(s) of the reference line(s) was (were) fuzzy, formed a spur, or did not curve clearly toward of the antigen well.

AGID readers

Only BLV-AGID analysts certified by the CFIA were involved in this study. For the European kits, a different pattern of punch had to be used. A proficiency panel composed of a quadruplicate set of 10 reference sera was set up for each European kit prior to the validation study and had to be read and interpreted by the BLV-AGID analysts. Two certified analysts independently interpreted all test results. A sample giving 2 positive readings was reported as positive, even if the degrees of positivity differed. In this case, the strongest reaction recorded among both readers was taken. Each positive sample was declared W, I, S, or VS. If both readings were negative, the sample was declared negative (N). In the case of a disagreement in interpretation, the sample was retested, the test was interpreted again by both analysts, and a final decision was taken from the last test. Those samples for which there was no consensus on the results of the second tests were declared doubtful (D). The data were entered into a spreadsheet (Microsoft Excel 97, Microsoft Corporation, Seattle, Washington, USA) and were analyzed.

Results

Disagreement on some results between both readers was found for all the kits used in this study. After the first test, a percentage of disagreement of 2.58% (American kit #1), 3.25% (European kit #2) and 7.25% (European kit #3), respectively, was observed. After the second test, these percentages dropped to 0.5% (American kit #1), 0.25% (European kit #2) and 1.08% (European kit #3), respectively. Tables I and II summarize the results obtained for the 1200 serum samples tested with the European kits #2 and #3, respectively, compared to the American kit #1, which was the reference standard. The European kit #2 detected 483 out of 490 expected positive samples and 701 out of 704 expected negative reactions, giving a total positive and negative agreement of 98.57% and 99.57% respectively. The kappa value for this kit was 0.99 (not considering the 9 doubtful samples). Similarly, the European kit #3 gave a total positive agreement of 471 out of 490 (96.12%) and a total negative agreement of 687 out of 704 (97.58%), with a kappa values of 0.96 (not considering the 19 doubtful samples). The data denote an excellent correlation between the American and both European AGID kits. A total positive agreement of 100% was found between both officially approved North American AGID test kits.

When the data were analyzed, 7 and 12 false negative samples were detected with the European kits #2 and #3, respectively. Of the false negative samples detected with the kit #2, all were found to be weakly positive (W) with the American kit #1. With the European kit #3, there were false negatives with W ($n = 7$), intermediate (I) ($n = 4$), or very strong (VS) ($n = 1$) positives. With kit #2, no false positives were seen. Kit #3 detected 11 false positives that were interpreted as W ($n = 6$), I ($n = 4$), or VS ($n = 1$) reactions.

For further in depth evaluation of the differences between the North American and European BLV-AGID kits, the degree of the positive reactions were compared. Table III summarizes the data obtained. Interestingly, all those samples found to be in the range of the I, S, or VS positive with the American kit #1 were also found to be positive (any degree of reaction) with the European kit #2; however, only 65% of the W samples were detected with the European kit #2. Similarly, kit #3 detected a very high percentage of the I, S, and VS, but only detected 50% of the W samples. The Canadian kit #4, however, detected all of the W, I, S, and VS positives ($n = 415$).

To determine the limit of detection of the BLV antibodies by each of the 3 kits, 40 strongly BLV-positive samples (with a degree of positivity of S or VS) and 10 negative samples were diluted from neat to 1/50 with the same batch of BLV-negative bovine serum. All of the negative sera were found to be negative at all the

Table III. Comparison of the number (percentage) of positive reactions with the European BLV-AGID kits in relation to the observed degree of positive reactions with the American BLV-AGID kit #1 from 490 positive samples

	American BLV-AGID kit #1	European BLV-AGID kit #2	European BLV-AGID kit #3
Degree of observed positive reaction	Number of samples	Number of samples (% of observed)	Number of samples (% of observed)
W	20	13 (65)	10 (50)
I	111	111 (100)	103 (92.8)
S	316	316 (100)	316 (100)
VS	43	43 (100)	42 (97.7)
Total	490	483 (98.6)	471 (96.1)

Table IV. Comparison of the American BLV-AGID kit #1, the European BLV-AGID kit #2 and the European BLV-AGID kit #3 on 40 strong positive samples diluted from neat to 1/50

Dilution	American kit #1 Number of positives (%)	European kit #2 Number of positives (%)	European kit #3 Number of positives (%)
Neat	40 (100)	40 (100)	39 (97.5)
1/5	40 (100)	39 (97.5)	38 (95.0)
1/10	38 (95.0)	34 (85.0)	30 (75.0)
1/25	27 (67.5)	19 (47.5)	3 (7.5)
1/50	18 (45.0)	6 (15.0)	0 (0)

dilutions with the 3 kits, with the exception of one sample that was declared doubtful at neat and at 1/5 dilutions with European kit #3. Table IV summarizes the data obtained for the positive samples. The American kit #1 detected more positive samples at all the dilutions.

Discussion

In this study, BLV-AGID kits commercially available either in North America or in Europe were compared. The North American kits and the European kits differed significantly. The punches used for these kits, which are standardized for their respective jurisdictions, were different. The wells of the North American kits were wider and were of the same diameter for the antigen, the reference serum, and the serum to be tested. In contrast, the wells of the European kits were smaller, with a different diameter for the antigen well compared with the reference and the test sample wells. The North American punch is currently the only one that is approved for the BLV-AGID kits used in Canada for official testing.

The pattern distribution of the reference and field samples also differed significantly. In the North American kits, of the 6 surrounding wells, 3 alternating wells are filled with the reference sera while the remaining wells are filled with the test sera. By contrast, in the European kits, only the top and bottom wells are filled with the reference sera, and the 4 outer wells are filled with the sera to be tested (compare Figures 1a and 1b). The consequence of these patterns was a different profile for the immunoprecipitation lines. There was only one reference line for each test sample for the European kits, as opposed to 2 reference lines with the North American kits. In order to read and interpret the European kits, the CFIA-certified readers had to carefully practice the European patterns with negative and positive (varying degrees) samples for which a common profile was established (refer to materials and methods) prior to the comparison study. According to the CFIA-certified analysts, the North American kits were easier to read and interpret, probably because the AGID readers rely on 2 immunoprecipitation lines with these kits, instead of one, as for the European kits. Also, the CFIA-certified analysts were used to interpret the reactions of the North American kits but not the reactions of the European kits. Ideally, the CFIA readers would have been trained and certified by European analysts, to ensure the difficulty in interpreting the European kits was not responsible for the apparent lack of sensitivity of these kits for the weak positive reactions. Among the European kits, it was found that kit #2 was easier to read and interpret than the kit #3, for which the percentage of disagreement between the 2 CFIA analysts was higher.

Based on the 1200 serum samples that were tested, an excellent correlation was found between the AGID kits. The kappa value was 0.99 for European kit #2 and 0.96 for European kit #3 when compared to the American kit #1 (not considering the doubtful reactions). However, significant differences were observed upon indepth analysis of the test results. When the positive samples were sorted according to their degree of positivity, a significant percentage of the W positive reactions detected by both North American kits were not detected by the European kits. The kappa values could have been lower if more W positive samples were included in the study. The selection of samples was done randomly among samples submitted for diagnostic purposes at our laboratory. Only 20 W positive out of 490 positive samples were selected, representing only 4.1% of the positive samples or 1.67% of the total number of samples. Most of the animals that were BLV-positive exhibited a strong immune response, as indicated by a percentage (73.3%) of the positive

samples being in the range of S or VS positives. The apparent lack of sensitivity of the European kits compared with the North American kits was confirmed by the dilution trial. In this experiment, S positive samples were diluted up to the limit of detection of the kits. The North American kit was still detecting antibodies in 45% of the samples diluted up to 1/50, while only 15% and 0% of the samples were detected at this dilution by the European kit #2 and #3, respectively.

Antibodies to BLV appear 3 to 12 wk following infection (11). Cattle infected with BLV are considered to be permanent carriers of the virus. Interpretation of the serological test is based upon the assumption that once an animal is infected, it remains infected for life. Weakly positive samples included in this study may have been from cattle that were recently infected and were in the process of mounting an antibody response to BLV. Also, serum antibody levels of BLV during the periparturient period are known to be reduced (12). In field situations, based on diagnostic data, BLV-infected cattle with low levels of antibodies, as indicated by serological assays, are less frequently observed than strong BLV reactors. It is nevertheless important to detect these animals by using sensitive assays because they remain potential reservoirs of BLV, and can thus infect cattle free of the disease.

It was proposed by Hoff-Jorgensen to standardize the diverse serological assays available for the diagnosis of BLV (13). A reference serum called E4 was selected to establish the harmonization among the AGID tests used in the western European countries and was adopted as an OIE reference standard (13). It was stated that the reference serum E4 diluted 1/10 in negative serum must be scored positive in the AGID test (13). In Canada, however, an AGID kit must pass a proficiency panel composed of a quadruplicate of 10 reference sera. All of the degrees of positive reactions are represented in this panel, including W positive samples.

Based on the overall results, it was found that there were different standards between the European and North American BLV-AGID kits. Not only the sensitivity of the 2 European kits, which, when evaluated, was found to be lower for the weak positive samples, but the punches, the amount of antigen and sera, the pattern of sample distribution, and the performance check based on reference sera were also different. Other tests, such as ELISA, that objectively measure antibody responses, must also be compared. A comparative study between the AGID and BLV-ELISA from the USA and Europe is presented in a companion paper.

Acknowledgments

The authors acknowledge Dr. Ann Wiegers for her help and comments on the establishment of the validation procedures. A special thanks to Paul Jorgensen and Brenda Gillcash for their help in the development (PJ) and the entering of information (BG) into the Excel database. Thanks also to Patricia Singh, Michèle Dunbar, and Christine Bélanger for their technical help, and to Drs. Stevenson and Gilles Dulac for reviewing the manuscript.

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