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SENSITIVITY AND SPECIFICITY OF A MODIFIED AGAR GEL PRECIPITATION TEST AND ITS APPLICATION TO THE DIAGNOSIS OF ENZOOTIC BOVINE LEUKOSIS

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Summary. — Sensitivity and specificity of a modified agar gel immuno-diffusion (AGID) test for the diagnosis of enzootic bovine leukosis was compared in 609 sera using the standard method, the modified test, and ELISA. The modifications concerned the composition of agar gel (1.8 % Bacto-agar Difco), addition of polyethylene glycol (PEG 6000, 4 %) and enlarging the diameters of the wells from 6 to 10 mm for sera. The change in well diameters and the addition of PEG 6000 increased the sensitivity of the test by about 60—100 %, favourably influenced the proper interpretation of the results, especially with weak positive sera. ELISA proved to be more sensitive either than the modified (by 4.1 %) or standard AGID (by 5.25 %) tests. The modified AGID test may find wide application in laboratory practice, especially when a more sensitive technique is not available.

Key words: *enzootic bovine leukosis*; *modified double diffusion test*; *polyethylene glycol 6000*; *ELISA*

Introduction

The development of the methods for multiplication of enzootic bovine leukosis virus (EBLV) in vitro (Chung *et al.*, 1984; van der Maaten, 1974 and 1976; Miller *et al.*, 1969) and the demonstration of the presence of specific antibodies in infected animals (Ferrer *et al.*, 1972; Miller and Olson, 1972) allowed the elaboration of specific serological diagnostic procedures. Among various tests introduced to laboratory practice, agar gel immunodiffusion (AGID) and enzyme linked immunosorbent assay (ELISA) have found general application. It is mainly due to the low costs of the tests, their relative simplicity, and high specificity, conditioned by the quality of the antigen used. In comparison with such serological tests as radioimmunoassay (RIA) or serum neutralization of BLV-VSV pseudotypes, they are much less time-consuming and do not require special equipment. Nevertheless, the AGID test, the easiest and cheapest of all the above mentioned

tests is less sensitive (Behrens *et al.*, 1979; Mammerickx *et al.*, 1980; Manz *et al.*, 1981; Graves *et al.*, 1982; Ban *et al.*, 1982; Mammerickx *et al.*, 1984; Liebermann *et al.*, 1985; Mammerickx *et al.*, 1985*a*; Mammerickx *et al.*, 1985*b*; Samól *et al.*, 1986; Hofirek *et al.*, 1986), although it may demonstrate the presence of antibodies since day 16 after artificial infection (Mammerickx *et al.*, 1980). Yet, in natural infection the ELISA test, due to its very high sensitivity, allows to reveal antibodies by 5 weaks earlier than the AGID test (Meyer *et al.*, 1986).

The aim of the present studies was the estimation of an improved and modified AGID test (Wawrzkiewicz *et al.*, 1986) for detection of antibodies to enzootic bovine leukosis (EBLV) in comparison with the standard ELISA procedure.

Materials and Methods

Antigens. Antigens purchased from Behringwerke AG, Marburg, series A 4180810A and A 418082A, and diagnostic preparations produced by the Veterinary Institute in Puławy (series No. S. 552 and A4319) were used.

Sera. 609 sera were taken from cattle in farms infested with EBLV (Wrocław province) and stored at −20 °C till use.

Agar gel immunodiffusion test (AGID). 1.8 % solution of Bacto-agar (Difco in Tris-HCl buffer, pH 7.2, containing 8.5 % NaCl and 4 % polyethylene glycol 6000 (PEG 6000), was poured onto Petri dishes. After solidification, wells were cut in diameter 6 or 10 mm at the distance of 5 mm from the edge of the central well (5 mm in diameter). A plate 80 mm in diameter was filled with 7 ml of dissolved agar gel to obtain a 2.5 mm thick gel layer. Control plates, prepared by standard procedures, had the same composition, except that the agar-agar concentration was 1.5 % and it contained no PEG 6000. The 5—6 peripheral wells (depending on the diameter), were filled with the sera to be examined, while the central well contained the appropriate antigen. The plates were kept at 22 °C in a humid chamber. Results were read after 24, 48 and 72 hr.

ELISA. ELISA test was performed according to the producer's instructions (Bioveta, národní podnik, Ivanovice na Hané, Č.S.S.R.). The antigen was dissolved in ELISA buffer and placed into the wells on a polystyrene plate. After incubation at 4 °C for 18 hr the diluted sera, beginning with 1 : 100, were added to the wells on the washed plate and incubated at 37 °C for 90 min. After washing, repeated four times, the wells were filled with the conjugate (50 µl) and again incubated at 37 °C for 90 min. Substrate solution (100 µl) containing 5-aminosalicylic acid and a fresh H_2O_2 were added and the reaction was read after incubation at room temperature for 60 min. The appearance of red-brown colour was regarded as positive. Each serum was tested twice in two adjoining wells.

Statistical analysis. The sensitivity of the AGID test in comparison with the ELISA test was estimated by χ^2 test, while the effects of various well sizes and the PEG 6000 addition on the results of the reaction — by Wilcoxon signed rank test.

Results

The data in Table 1 indicate that application of the agar gel immunodiffusion test in our own modification consisting in enlarging the diameter of the wells from 6 to 10 mm and in an addition of polyethylene glycol (PEG 6000) in the concentration of about 4 %, has increased the sensitivity of the test since the number of positive results increased from 385 to 392 (1.15 %). The number of uncertain results also increased from 17 to 22 (0.82 %). The highest sensitivity was found by the ELISA test which detected antibodies to EBLV in the most of sera examined. In comparison with the modified

Table 1. Comparison of the sensitivity of the standard and modified AGID tests

Farm	Group of animals	No. of animals (%)	Modified AGID test			Standard AGID test*			ELISA test		
			Results								
			+	−	±	+	−	±	+	±	±
W	Cows	212	183 (86.32)	17 (8.02)	12 (5.06)	179 (84.43)	21 (9.91)	12 (5.66)	192 (90.57)	16 (7.55)	4 (1.88)
	Cows	78	56 (71.79)	18 (23.08)	4 (5.13)	54 (69.23)	22 (28.21)	2 (2.56)	62 (79.49)	14 (17.96)	2 (2.56)
D	Heifers	8	2 (25.00)	5 (62.50)	1 (42.50)	2 (25.00)	6 (75.00)	— —	3 (37.50)	5 (62.50)	— —
J	Heifers	120	41 (34.17)	78 (65.00)	1 (0.83)	41 (34.17)	78 (65.00	1 (0.83)	44 (36.67)	76 (63.33)	— —
N	Heifers	73	17 (23.29)	53 (72.60)	3 (4.11)	16 (21.29)	56 (76.71)	1 (1.37)	19 (26.03)	54 (73.97)	— —
	Cows	116	92 (79.31)	23 (19.83)	1 (0.86)	92 (79.31)	23 (19.83)	1 (0.86)	96 (82.76)	19 (16.38)	1 (0.86)
Wk	Heifers	2	1 (50.00)	1 (50.00)	— —	1 (50.00)	1 (50.00)	— —	1 (50.00)	1 (50.00)	— —
	Cows	406	331 (81.53)	58 (14.28)	17 (4.19)	325 (80.05)	66 (16.26)	15 (3.69)	350 (86.21)	49 (12.07)	7 (1.72)
	Heifers	203	61 (30.05)	137 (67.49)	5 (2.46)	60 (29.56)	141 (69.46)	2 (0.98)	67 (33.00)	136 (67.00)	— —
	Total	609	392 (64.37)	195 (32.02)	22 (3.61)	385 (63.22)	207 (33.99)	17 (2.79)	417 (68.47)	187 (30.38)	7 (1.15)

* Behringwerke, AG

Table 2. Relationship between the size of wells, the composition of agar gel and the appearance of precipitation lines

No. of the serum	ELISA titre	Agar gel + 4 % of PEG				Agar gel without PEG			
		Large wells		Small wells		Large wells		Small wells	
		Behr.*	Inst. Wet.**	Behr.*	Inst. Wet.**	Behr.*	Inst. Wet.**	Behr.*	Inst. Wet.**
1	200	1 : 4	1 : 4	1 : 8	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4
3	200	1 : 2	1 : 2	1 : 4	1 : 2	1 : 2	1 : 2	1 : 2	1 : 1
12	200	1 : 8	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4
18	200	1 : 8	1 : 8	1 : 4	1 : 4	1 : 4	1 : 8	1 : 4	1 : 4
20	200	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 2	1 : 2
53	200	1 : 2	1 : 2	1 : 2	1 : 1	1 : 2	1 : 2	1 : 1	1 : 1
55	200	1 : 2	1 : 2	1 : 2	1 : 2	1 : 2	1 : 2	1 : 1	1 : 1
5	400	1 : 16	1 : 16	1 : 8	1 : 8	1 : 8	1 : 8	1 : 8	1 : 8
4	400	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 2	1 : 2
19	400	1 : 8	1 : 8	1 : 8	1 : 8	1 : 8	1 : 8	1 : 4	1 : 4
31	400	1 : 2	1 : 2	1 : 1	1 : 1	1 : 1	1 : 1	1 : 1	1 : 1
34	400	1 : 8	1 : 8	1 : 4	1 : 8	1 : 4	1 : 4	1 : 1	1 : 2
43	400	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 1	1 : 2
57	400	1 : 8	1 : 12	1 : 8	1 : 8	1 : 8	1 : 8	1 : 1	1 : 2
59	400	1 : 4	1 : 4	1 : 2	1 : 2	1 : 2	1 : 2	1 : 1	1 : 1
26	800	1 : 12	1 : 12	1 : 8	1 : 8	1 : 8	1 : 8	1 : 8	1 : 4
46	800	1 : 16	1 : 16	1 : 12	1 : 8	1 : 12	1 : 16	1 : 4	1 : 8
6	800	1 : 12	1 : 8	1 : 8	1 : 8	1 : 8	1 : 8	1 : 4	1 : 4
37	800	1 : 12	1 : 16	1 : 12	1 : 8	1 : 12	1 : 12	1 : 4	1 : 8
38	800	1 : 12	1 : 12	1 : 8	1 : 12	1 : 8	1 : 8	1 : 4	1 : 6
42	800	1 : 8	1 : 8	1 : 8	1 : 8	1 : 8	1 : 8	1 : 8	1 : 8
	1600	1 : 30	1 : 30	1 : 20	1 : 20	1 : 30	1 : 25	1 : 16	1 : 8
9	1600	1 : 40	1 : 32	1 : 20	1 : 20	1 : 40	1 : 40	1 : 20	1 : 20
174	1600	1 : 40	1 : 25	1 : 25	1 : 16	1 : 40	1 : 25	1 : 25	1 : 25
216	1600	1 : 30	1 : 30	1 : 20	1 : 20	1 : 30	1 : 30	1 : 16	1 : 16
SG (mean geometric		7.955	7.613	6.195	5.664	6.342	6.426	3.478	3.737

* Antigen manufactured by Behringwerke AG
** Antigen manufactured by Instytut Weterynarii in Puławy

precipitation test the number of positive results was higher by 4.1 % and, in relation to the standard procedure, by 5.25 %.

The data in Table 2 show that addition of PEG 6000 to the agar raised the sensitivity of the test regardless of the antigen used. Mean geometric titres were 7.95 as compared to 6.34 with antigen BW, and 7.613 and 6.426 with antigen IW, respectively. The results, additionally estimated by means of Wilcoxon signed rank test, confirmed the statistically significant differences ($P \leq 0.05$) in the case of BW antigen. In a pattern of small wells, the data were 6.195 and 3.478 for antigen BW and 5.664 and 3.737 for antigen IW, respectively. The differences were statistically significant ($P \leq 0.05$) for both antigens.

Table 3. AGID test performed with diluted antigens

No. of the serum	Titre in ELISA test	Agar gel + 4 % of PEG		Agar gel without PEG	
		Behr.*	Inst. Wet.**	Behr.*	Inst. Wet.**
12	200	1 : 4	1 : 4	1 : 3	1 : 3
3	200	1 : 5	1 : 6	1 : 5	1 : 5
20	200	1 : 5	1 : 5	1 : 4	1 : 4
5	400	1 : 2	1 : 3	1 : 2	1 : 2
4	400	1 : 3	1 : 3	1 : 1	1 : 1
31	400	1 : 4	1 : 4	1 : 4	1 : 4
26	800	1 : 4	1 : 4	1 : 3	1 : 3
37	800	1 : 2	1 : 2	1 : 1	1 : 1
46	800	1 : 3	1 : 4	1 : 3	1 : 3
24	1600	1 : 3	1 : 3	1 : 1	1 : 1
	Geometric mean	3.3401	3.6457	2.3097	2.3097

* Antigen manufactured by Behringwerke AG
** Antigen manufactured by Instytut Weterynarii in Puławy

The value of the antigens examined was additionally estimated by the dilution method, using an agar medium with addition of PEG 6000 or without it, and of a pattern in which peripheral wells 6 mm in diameter were filled up with successive dilutions of the antigen while the central well, 10 mm in diameter, was filled with serum (Table 3). It has turned out that, regardless of the antigen used, addition of PEG 6000 to the agar exerted an advantageous influence on the sensitivity of the precipitation reaction. The mean geometric titres were from 3.34 to 3.64 in comparison with the mean geometric titre of 2.31 in the case of a medium without polyethylene glycol. The statistical analysis by Wilcoxon signed rank test confirmed that the differences were statistically significant, regardless of the diagnostic antigen used. At the same time, they demonstrated the lack of significant difference between antigen BW and antigen IW in respect to the value of their reaction to antibodies in the form of precipitation lines.

Discussion

The technique of double diffusion in gel, designed by Ouchterlony (1958), still finds wide application in the diagnosis of chronic virus diseases, especially in identifying enzootic leukosis in cattle. Yet, the sensitivity of this test, as compared with that of the ELISA, is much lower and therefore makes it impossible to assess all infected animals, namely those in the early period postinfection or in the case of a considerable decrease of antibody level in the perinatal period (Burridge *et al.*, 1985). In order to improve the test, two modifications were introduced; they both proved advantageous by raising

the sensitivity of the test. The proposed medium composition allowed to demonstrate low concentrations of antibodies in animal sera.

The lack of a visible precipitation reaction in the gel at relatively low antibody levels is generally known. It is in full agreement with the fact that the classical AGID test reveals lower percentage of seropositive animals than radioimmunoassay (Mammerickx *et al.*, 1980), ELISA test (Behrens *et al.*, 1979; Portetelle *et al.*, 1983; Mammerickx *et al.*, 1984; Mammerickx *et al.*, 1985; Samól *et al.*, 1986) or serum neutralization of BLV-VSV pseudotypes (Zajac *et al.*, 1980). In this connection Banneberg (1982; 1986) designed a different pattern of arrangement (3 : 3, instead of 4 : 2) demonstrating an increase in the number of positive reactions in the first arrangement avoiding at the same time erroneous false positive reactions. However, his method requires the use of control serum in not fewer than 3 out of 6 wells employed. Our modification does not increase the number of wells with control sera and makes it possible to read the result correctly already within 24—48 hr; exceptionally positive reactions may appear after 3 days only. A low antibody level antigen—antibody complex is initially invisible, but prolonged storage of the plates does not affect the percentage of positive results.

Introduction of polyethylene glycol (PEG 6000) as determined in our previous experiments, in concentrations of 1 %, 2 %, and 3 % did not clearly influence the formation of precipitation lines, while the concentration of 5 % caused a strong opacity of the medium as well as unfavourable changes in the gel consistency. For that reason, in accordance to Kostner and Holasek (1972) who used PEG for immunoelectroprecipitation, we assumed that addition of PEG in the amount of 4 % was the most appropriate. We increased the concentration of agar from 1.5 % to 1.8 % in order to improve gel consistency. Initial experiments with this changed gel using calf serum as antigen and rabbit serum as antibody have shown typical precipitation lines at twice as high dilution of immune serum than in the absence of PEG. These data are in agreement with those of Kostner and Holasek (1972) who first suggested to use PEG 6000 and dextran 70 for immunoelectroprecipitation and noticed a five-fold increase in the sensitivity of the test. Our present studies, however, showed only a slightly higher sensitivity of the AGID test after introduction of PEG 6000 to the gel, but it should be emphasized that the assay was different as well as the antigen which must have undoubtedly affected the final results. Nevertheless, there can be no doubt that an addition of PEG to agar gel favourably influenced the precipitation reaction, especially when the sera contained low levels of antibodies or when a low concentration of antigen had been applied. The phenomenon is probably connected with a lowered degree of solubility and better precipitation of the antigen — antibody complexes.

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