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Epidemiological evaluation of a monoclonal ELISA detecting antibodies against bovine leukaemia virus in serum pools

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SUMMARY

Sensitivity, specificity and predictive values of an enzyme-linked immunosorbent assay (ELISA) for detecting antibodies against bovine leukaemia virus (BLV) were evaluated using a representative sample of 145 serum pools, comprising from 3 to 48 individual sera. The sample was constituted according to the frequency distribution of the negative and positive pools analysed during a screening involving the whole cattle population of Belgium. Sensitivity and specificity were estimated to 88·9% and 100% and the predicted negative and positive values were 99·9% and 100%, respectively. These results indicate the use of serum pools is suitable for the detection of BLV infected herds in eradication campaigns.

INTRODUCTION

The agent of enzootic bovine leukosis (EBL) is an exogenous retrovirus (bovine leukaemia virus, BLV) which is transmitted horizontally [1]. The disease is characterized by a long latency period following initial infection, to the development of tumours which occurs in up to 10% of infected animals. During the latency period the only evidence of infection is the presence of antibodies directed against viral proteins, essentially the gp51 envelope glycoprotein and the major core protein p24. Antibodies against gp51 appear earlier than those against p24 and are usually present at a higher titer [2, 3].

An indirect ELISA, based on the recognition of gp51 antibodies, was used in the eradication campaign conducted in Belgium between 1989–91 [4]. The use of ELISA on serum pools allowed important financial saving since 89301 serum pools were analysed during the Belgian screening programme instead of 2027413 individual sera constituting the pools [4].

During the national survey, 88742 serum pools were negative and 559 positive for BLV antibodies. Farms with a positive serum pool were sampled a second time and an immunodiffusion test performed on individual sera in compliance with the Directive 88/406/EEC (OJ No L 194, 22 July 1988). The negative status of the other farms was confirmed by two consecutive herd tests carried out at an interval of approximately one year.

The performance of ELISA applied to serum pools has not been investigated for BLV infection. However, it is of importance to establish the sensitivity, specificity and predictive value of the test if it is to be used in eradication programmes, especially when the prevalence of the infection is low, as it was in Belgium where the prevalence was estimated at 1·34% in the screening programme carried out during 1983–4 and 1984–5 [5].

Only the relative sensitivity and specificity of the ELISA had been estimated before this study. Mammerickx and colleagues [6] detected 26 false positives out of 26000 pools of 75 sera. Based on these results the ELISA test was improved by using an anti-bovine IgG1 monoclonal antibody [7]. The modified ELISA test was used first with sera from experimentally inoculated animals and reference sera [7] and then used during the Belgian eradication campaign [4].

The purpose of this investigation was to determine the true sensitivity, specificity and predictive values of the ELISA test as used in serum pools for the screening of BLV in Belgium. The whole Belgian cattle population was screened during a 3-year period using serum pools. A satisfactory representative sample of the population was obtained by distributing the numbers of individual sera constituting each pool in this study according to the frequency distribution of the numbers of individual sera constituting the 89301 pools submitted for the screening programme.

METHODS

ELISA

A modification of an indirect ELISA employing two monoclonal antibodies was used [7]. The first antibody bound the BLV gp51 present in an unpurified virus preparation produced from culture supernatant of a fetal lamb kidney (FLK) cell line infected with BLV while an anti-bovine IgG1 monoclonal antibody [8] was used as the conjugate. The enzyme used was β galactosidase with orthonitrophenyl-β-D-galactoside (ONPG) as the substrate.

The modifications were as follows: the serum pools were tested at dilutions of 1:20 and 1:60; 200 μl of each dilution being added to each well. After an overnight incubation at 4 °C, the plates were washed (SLT Labinstrument EAW II plus) with PBS (sodium phosphate 0·01 M, NaCl 0·15 M, pH 7·4) containing 0·2% Tween-80 (washing buffer) and 50 μl of conjugate added to each well. After 3 h incubation at room temperature, the plates were washed with washing buffer and 100 μl of substrate added. The light absorbance was measured with a spectrophotometer (LP 400, Diagnostic Pasteur, Paris) at 405 nm and 620 nm. When the positive control reached the predetermined optical density (OD) the reaction was stopped by addition of 100 μl Na_2CO_3 1 M per well. The samples presenting an OD higher than 0·150 at the dilution 1:60 were considered positive.

Composition of serum pools

Between 1989 and 1991, 89301 serum pools were screened for BLV infection using ELISA. Of these, 559 (0·63%) pools were positive. The number of sera comprising the negative and positive pools are listed in Tables 1 and 2. Herds identified as positive by ELISA were blood tested a second time and the sera from each animal tested using an immunodiffusion test in compliance with Directive

Table 1. *Frequency table of the screened and constructed negative serum pools according to the number of sera in the pool*

Number of sera in the pool	Frequency of the screened serum pools	Frequency of the constructed serum pools
1–5	20765	23
6–10	9491	11
11–15	6395	7
16–20	5988	7
21–25	5716	6
26–30	10274	12
31–35	6513	7
36–40	10345	12
41–45	4334	5
46–50	8921	10
Total	88742	100

Table 2. *Frequency table of the screened and constructed positive serum pools according to the number of sera in the pool*

Number of sera in the pool	Frequency of the screened serum pools	Frequency of the constructed serum pools
1–5	37	3
6–10	34	3
11–15	34	3
16–20	37	3
21–25	32	3
26–30	90	7
31–35	51	4
36–40	112	9
41–45	42	3
46–50	90	7
Total	559	45

88/406/EEC. The numbers of positive animals in the 559 pools, according to the numbers of sera in the pool tested by ELISA, are given in Table 3.

The information provided in Tables 1–3 was used to construct 145 pools of sera to validate the ELISA. The negative sera were derived from herds which were negative for BLV on at least two occasions at an interval of one year using the ELISA as the screening test, while the positive sera were from cattle positive using the immunodiffusion test. All these sera were stored at −20 °C until used.

The numbers of positive and negative sera in each constructed pool are listed in Tables 1 and 2 and were chosen according to the central values of each class. The frequency distributions of the negative and positive constructed serum pools were calculated by multiplying the relative frequency of each class by 100 and 45, respectively. The frequency of positive sera in the 45 positive constructed serum pools (Table 4) was calculated by multiplying, for each class, the relative frequency of Table 3 by the frequency of the constructed serum pools of Table 2. A regrouping of some of the classes was necessary to obtain whole numbers.

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Table 3. *Frequency of the number of positive individuals from each group of the 559 BLV-positive pooled sera*

Number of positive sera	Number of sera in the pool									
	1–5	6–10	11–15	16–20	21–25	26–30	31–35	36–40	41–45	46–50
1	24	21	14	18	12	28	17	33	14	18
2	8	5	8	6	10	16	7	18	6	17
3	4	2	5	2	2	10	4	14	1	8
4	1	1	4	3	1	6	6	10	4	6
5	—	4	—	1	3	7	2	1	1	7
6	—	1	—	—	2	3	3	4	1	4
7	—	—	1	3	—	7	4	8	1	8
8	—	—	1	—	1	1	—	2	3	3
9	—	—	1	2	—	5	—	—	1	1
10	—	—	—	1	—	1	—	4	1	3
11	—	—	—	—	1	1	—	—	—	3
12	—	—	—	—	—	—	—	2	—	—
13	—	—	—	—	—	—	1	2	1	1
14	—	—	—	1	—	1	1	—	—	1
15	—	—	—	—	—	—	2	3	—	—
16	—	—	—	—	—	2	—	—	—	1
17	—	—	—	—	—	2	—	—	1	1
18	—	—	—	—	—	—	—	4	—	1
19	—	—	—	—	—	—	—	—	—	—
20	—	—	—	—	—	—	1	2	1	—
21	—	—	—	—	—	—	—	—	2	—
22	—	—	—	—	—	—	—	—	1	2
23	—	—	—	—	—	—	—	—	1	—
24	—	—	—	—	—	—	—	—	—	—
25	—	—	—	—	—	—	1	1	—	1
26	—	—	—	—	—	—	1	3	—	—
27	—	—	—	—	—	—	1	1	1	—
28	—	—	—	—	—	—	—	—	—	—
29	—	—	—	—	—	—	—	—	—	1
30	—	—	—	—	—	—	—	—	—	1
31	—	—	—	—	—	—	—	—	—	—
32	—	—	—	—	—	—	—	—	—	—
33	—	—	—	—	—	—	—	—	—	—
34	—	—	—	—	—	—	—	—	—	—
35	—	—	—	—	—	—	—	—	—	1
36	—	—	—	—	—	—	—	—	—	—
37	—	—	—	—	—	—	—	—	1	—
38	—	—	—	—	—	—	—	—	—	1
Total	37	34	34	37	32	90	51	112	42	90

The frequency of pools containing only 1–5 individual sera was high (essentially negative pools). This can be explained by the fact that samples either originated from small farms with one or a few animals and that sera from farms with more than 50 animals were usually divided with the first pool containing 50 sera and a second pool the remaining sera.

Evaluation of the ELISA

The specificity (*sp*), the sensitivity (*se*) and the empirical predicted positive value (*PPVe*) and negative value (*PNVe*) of ELISA were determined from a

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Table 4. *Frequency of the number of positive individuals from each group of the 45 BLV-positive constructed pooled sera*

Number of positive sera	Number of sera in the pool									
	3	8	13	18	23	28	33	38	43	48
1	2	2	1	1	1	2	1	2	1	1
2	1	—	1	—	1	1	1	1	—	1
3	—	—	—	1	—	1	—	1	—	1
4	—	1	—	—	—	—	—	—	1	1
5	—	—	—	—	1	1	1	1	—	—
6	—	—	—	—	—	—	—	—	—	—
7	—	—	1	—	—	1	—	1	—	—
8	—	—	—	—	—	—	—	—	—	1
9	—	—	—	1	—	—	—	—	—	—
10	—	—	—	—	—	—	—	1	—	—
11	—	—	—	—	—	1	—	—	—	—
12	—	—	—	—	—	—	—	—	—	—
13	—	—	—	—	—	—	—	—	—	—
14	—	—	—	—	—	—	—	—	—	1
15	—	—	—	—	—	—	1	—	—	—
16	—	—	—	—	—	—	—	—	—	—
17	—	—	—	—	—	—	—	—	—	—
18	—	—	—	—	—	—	—	1	—	—
19	—	—	—	—	—	—	—	—	—	—
20	—	—	—	—	—	—	—	—	1	—
21	—	—	—	—	—	—	—	—	—	—
22	—	—	—	—	—	—	—	—	—	—
23	—	—	—	—	—	—	—	—	—	—
24	—	—	—	—	—	—	—	—	—	—
25	—	—	—	—	—	—	—	—	—	—
26	—	—	—	—	—	—	—	1	—	—
27	—	—	—	—	—	—	—	—	—	—
28	—	—	—	—	—	—	—	—	—	—
29	—	—	—	—	—	—	—	—	—	1
Total	3	3	3	3	3	7	4	9	3	7

contingency table according to the following formulae [9]: $sp = d/(b+d)$; $se = a/(a+c)$; $PPVe = a/(a+b)$ and $PNVe = d/(c+d)$.

The true prevalence (P) of the infection was estimated from the apparent prevalence (Pa) of BLV positive herds in Belgium using the formula [10]: $P = [Pa+(sp-1)]/[se+(sp-1)]$.

Bayes's theorem [11] was used to determine the predicted positive value (PPV) and negative value (PNV) of ELISA by taking into account the estimation of the true prevalence. The formulae were as follows: $PPV = (P.se)/[(P.se)+[(1+P).(1-sp)]]$ and $PNV = [(1-P).sp]/[[(1-P).sp]+[P.(1-se)]]$.

RESULTS

All the 100 negative serum pools and 5 of the 45 serum pools containing positive sera were negative by the ELISA. These results gave 100 true negatives ($TN = d$), no false positive ($FP = b$), 40 true positives ($TP = a$) and 5 false negatives ($FN = c$). The 5 false negative pools comprised (expressed as number of positive sera/number of individual sera): 1/23, 1/28, 1/38, 2/38, 1/43.

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Table 5. *Contingency table with classification of ELISA results*

	BLV+	BLV−	
ELISA+	a = 40	b = 0	a+b = 40
ELISA−	c = 5	d = 100	c+d = 105
	a+c = 45	b+d = 100	a+b+c+d = 145

BLV+, positive serum pool: the BLV positive status is obtained after testing individual sera by immunodiffusion; BLV−, negative serum pool obtained from herds negative on at least two occasions and possessing the L3 status; a, true positives; b, false positives; c, false negatives; d, true negatives.

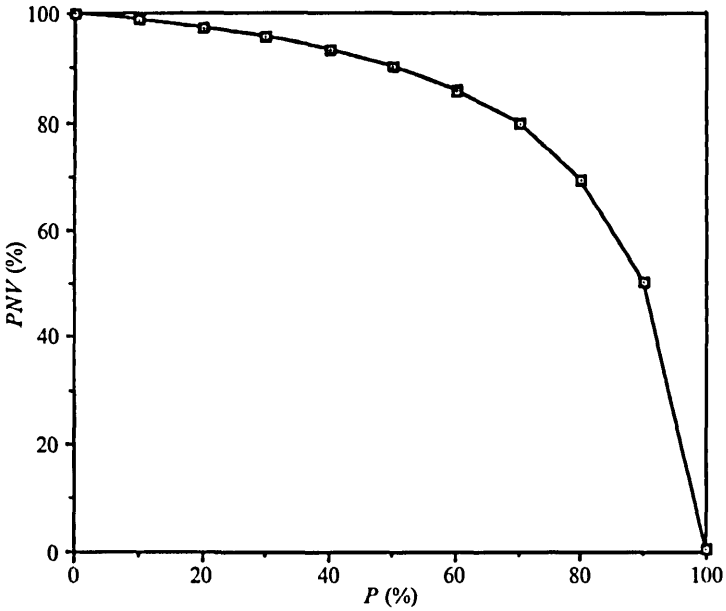

Fig. 1. Predicted negative value (*PNV*) of ELISA at different values of BLV prevalence (*P*).

By inference from the contingency table (Table 5), *se* was equal to 88·9%, *sp* to 100%, *PNVe* to 95·2% and *PPVe* to 100%. The actual prevalence (*P*) of BLV positive herds in Belgium was estimated from the apparent prevalence (*Pa*) calculated by Knapen and colleagues [4] and from the sensitivity and the specificity of the ELISA. *P* was equal to 1·1%. From Bayes's theorem, *PNV* was estimated at 99·9% and *PPV* at 100%. Figure 1 gives the different values of *PNV* calculated for different *P* values.

DISCUSSION

The evaluation of our ELISA for use in an EBL eradication programme can be achieved by either calculating of the agreement between the ELISA and a 'standard' test such as the immunodiffusion test or by calculating the sensitivity, specificity and predictive values with samples coming from herds with known BLV status. In the event only the second was applicable because in the Belgium screening programme the ELISA was used on serum pools and the

immunodiffusion test was performed on individual sera. As a consequence the evaluation of the ELISA had to be performed retrospectively, i.e. after its use in an eradication campaign. This was achieved by the creation of a relatively small number of pools which were representative of the whole cattle population since sensitivity and specificity are independent of prevalence [12].

The sensitivity and the specificity were 88·9% and 100% respectively which confirmed that the ELISA performed satisfactory in the eradication campaign. Moreover, the results described fit with those of Knapen and colleagues [4] who estimated empirically the sensitivity and the specificity of the ELISA to be 89·5% and 99·9%, respectively, based on compilation of the observed results in the field.

One of the objectives of this study was to define the predictive values of the ELISA test. The prerequisite for determining these values is to know the true sensitivity and specificity of the test which together with a knowledge of the observed prevalence allowed the estimation of the predictive values. For example, in a theoretical situation where the prevalence of BLV positive herds is as high as 60%, the *PNV* still remains higher than 85%. Therefore, the evaluation of the ELISA test proved that it was efficient when applied on serum pools and that reliability of the results was not diminished.

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