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RESEARCH ARTICLE

Comparison Between Four Laboratory Tests for Routine Diagnosis of Enzootic Bovine Leukosis

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Abstract

The aim of this study was to compare the diagnostic capabilities of the agar gel immunodiffusion test (AGID) and two types of PCR, nested PCR (with nucleic acid extraction from blood) and direct blood nested PCR (db nested PCR) - without extraction, vs. enzyme-linked immunosorbent assay (ELISA) as the gold standard in the routine diagnostics of this disease. A total of 409 blood samples were obtained from cattle 18 mo. to 5 yrs. of age, and all the samples were analyzed using the four assays. Following the initial testing, the samples were stored at -20°C and re-tested using all the four techniques after a month of freeze storage to determine the reproducibility of the results. ELISA detected 57 animals as positive (13.9%) versus 33 ones using AGID and 56 using the two types of nested PCR. AGID showed low sensitivity of 57.9% and moderate agreement compared to ELISA. In addition, AGID did not show consistency in the results from the two independent measurements. The two types of nested PCR showed nearly full agreement with ELISA with a kappa value of 0.99. Since AGID showed lower sensitivity and lack of reproducibility in the results for 22 samples as compared to the other techniques used in this study, we suggest that the future application of this test for the diagnosis of Enzootic bovine leucosis in blood samples should be reconsidered. On the other hand, db nested PCR demonstrated very good sensitivity and reproducibility of results, it also requires less sample processing. All this makes it potentially suitable for routine diagnostics.

Keywords: *AGID, db nested PCR, ELISA, Enzootic bovine leukosis, Nested PCR*

Enzootik Sığır Lökozunun Rutin Teşhisi İçin Kullanılan Dört Laboratuvar Testinin Karşılaştırılması

Öz

Bu çalışmanın amacı, agar jel immünodifüzyon testi (AGID) ve iki tip PCR'nin, nested PCR (kandan nükleik asit ekstraksiyonu ile birlikte) ve kanda direkt nested PCR (db nested PCR) - ekstraksiyon olmaksızın, tanısal yeteneklerinin bu hastalığın rutin teşhisinde altın standart olarak kullanılan Enzyme-Linked Immunosorbent Assay (ELISA) ile karşılaştırılmasıydı. Çalışmada, 18 ay ile 5 yaş arası sığırlardan toplam 409 kan örneği toplandı ve tüm örnekler bu dört yöntem ile analiz edildi. İlk analizleri takiben, örnekler -20°C'de saklandı ve sonuçların tekrarlanabilirliği açısından bir aylık dondurularak depolamanın ardından dört yöntem ile yeniden analiz edildi. ELISA ile 57 (%13.9) hayvan pozitif saptanırken, AGID ile 33 ve her iki tip nested PCR ile 56 hayvan pozitif saptandı. ELISA'ya kıyasla AGID, %57.9'luk düşük sensitivite ve orta düzeyde uyum gösterdi. Ayrıca AGID, iki bağımsız ölçümden elde edilen sonuçlarla da tutarlılık göstermedi. İki tip nested PCR yöntemi de 0.99 kappa değeriyle ELISA ile neredeyse tam uyum gösterdi. Bu çalışmada kullanılan diğer tekniklere kıyasla AGID, 22 örnekte daha düşük sensitivite gösterdiği için ve tekrarlanabilirliği olmadığı için kan örneklerinde Enzootik sığır lökozunun teşhisinde bu testin gelecekte uygulanmasının yeniden düşünülmesi gerektiğini öneriyoruz. Diğer taraftan, db nested PCR çok iyi bir sensitivite ve tekrarlanabilirlik gösterdi, ayrıca daha az örnek işlenmesine ihtiyaç duydu. Bütün bunlar, db nested PCR'yi rutin teşhis için potansiyel olarak uygun hale getirmektedir.

Anahtar sözcükler: *AGID, db nested PCR, ELISA, Enzootik sığır lökozu, Nested PCR*

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Introduction

Enzootic bovine leukosis (EBL) is a well-known infectious disease in cattle worldwide. Natural infection has been shown in buffalo, zebu and capybara in some regions [1,2]. EBL is caused by an oncovirus, bovine leukemia virus (BLV), which belongs to the *Deltaretrovirus* genus of the *Retroviridae* family [3]. BLV has a strong affinity for B-lymphocytes, but also infects other cells involved in the immune defenses. Owing to the oncogenic properties of the causal agent and the immune response imbalance, animals develop tumours of various clinical manifestation depending on their location [4]. Reportedly, lymphosarcoma develops in up to 10% of infected cattle, whereas persistent lymphocytosis, in 30 to 70% of animals [5]. In most cases, the integration of viral RNA in the form of proviral DNA in the host cell genome results in asymptomatic infection which facilitates the spread of the pathogen among sensitive populations [1]. The modes of BLV transmission are well known. The current understanding is that the major mode of transmission is horizontal and that the sources of infection in herds are animals with a proven high viral load [6,7].

Besides the above-mentioned natural hosts, some other animal species are susceptible to experimental infection, sheep being most sensitive and developing tumors at a younger age than cattle [8]. Studies have investigated the association between BLV and mammary cancer in human. Giovanna et al.[9] and Buehring et al.[10] found that the presence of proviral DNA in breast tissue was associated with a neoplastic process, whereas Zhang et al.[11] observed no association between BLV and breast cancer in Chinese women. Another study from Iran reported a possible association between BLV and development of some types of lymphoma in humans [12].

Enzootic bovine leukosis causes serious direct and indirect losses to farmers in countries with developed cattle breeding [13,14]. Early detection of infected animals using modern diagnostic approaches is essential for the management of EBL in cattle farms. Agar gel immunodiffusion (AGID) and enzyme-linked immunosorbent assay (ELISA) have been approved by the OIE as techniques for detection of anti-BLV antibodies in serum or milk [15]. In cases when antibody titers are very low or absent, e.g. in young calves with colostrum antibodies, different types of polymerase chain reaction (PCR) assays have been developed to detect proviral DNA in samples from virus-infected animals, including tumor tissue [16-19]. One of the innovations in EBL diagnosis is the use of PCR without prior DNA extraction [20]. This prompted us to adapt the nested PCR recommended by the OIE [15], as well as db nested PCR by omitting the proviral DNA extraction step from whole blood.

The introduction of state disease control programs based on diagnostic methods together with culling of positive animals has led to limitation or complete elimination of EBL in a lot of Western European countries, Scandinavia and Oceania [21]. There have been reports of high seroprevalence in North and South America, Eastern Europe and Asia [7,22]. The seroprevalence in Bulgaria varied from 0.00% to 63.85% in different regions in 2012, according to Sandev et al.[23].

The aim of this study was to perform comparative analysis of the diagnostic capabilities of laboratory tests, agar gel immunodiffusion test (AGID), nested PCR and db nested PCR vs. enzyme-linked immunosorbent assay (ELISA) as the gold standard in the routine diagnostics of EBL.

Material and Methods

A total of 409 blood samples were collected from cattle 18 months to 5 years. of age. The animals were reared in farms in the South-Central Region of Bulgaria. The blood samples were tested using four laboratory assays. All samples were taken aseptically by jugular vein puncture and were collected in Vacutainer™ collection tubes with EDTA K3 anticoagulant (Wenzhou Gaode Medical Instrument, China) for proviral DNA extraction and in sterile Serum Blood collection tubes, 5 mL (Wenzhou Gaode Medical Instrument, China) for the serological assays.

Agar Gel Immunodiffusion

The Bovine Leukosis POURQUIER AGID test (Institut Pourquier, France) was used for detection of anti-gp51 antibodies, according to the manufacturer's instructions. Petri dishes loaded with the components: antigen, positive controls and test samples, were incubated at 22°C in a humid camera and were inspected for lines of precipitation every 24 h over 3 days. The sera were re-assayed independently following storage at -20°C for 1 month.

Enzyme Linked Immunosorbent Assay (ELISA)

A competitive ELISA kit was used for the detection of antibodies against the gp51 envelope protein of BLV (IDVet, France). According to the manufacturer's instructions, we followed the brief procedure, with initial incubation at 21°C±5°C for 45 min ± 4 min. The optical density (OD) values were read at 450 nm using a microplate photometer (Biosan, Latvia). The results were validated and interpreted according to the following criteria and equations in the manufacturer's instructions:

Validation: mean value of negative controls (2 wells C1 and D1) higher than 0.7 (OD_{NC}>0.7); mean value of positive controls (OD_{PC}) (2 wells A1 and B1) at least 30% that of the negative control (OD_{NC}), or OD_{PC}/OD_{NC} <0.3;

Interpretation: Competition % = (OD_{SAMPLE}/OD_{NC}) x 100 *(Table 1)*.

Table 1. Interpretation of competitive ELISA results	
Result	**Status**
% Competition ≤ 50%	Positive
50 < % Competition < 60%	Doubtful
% Competition ≥ 60%	Negative

The sera were re-assayed using ELISA independently following storage at -20°C for 1 month.

Nested PCR Assay

Proviral DNA was obtained from whole blood (100 µL) using the ISOLATE II Genomic DNA Kit (Bioline, UK). PCR was performed in 25 µL reaction volume: 12.5 µL MyTaq red PCR mix (Bioline, UK), 3.0 µL DNA, 1 µL primers (produced by Jena Bioscience, Germany) each in a working concentration of 10 pmol/µL and 7.5 µL molecular biology grade water (Bioline, UK). The reactions were run in a Quanta Biotech Termal Cycler (Quanta Biotech, UK) with the following temperature profile for the first-round PCR: denaturation at 95°C for 3 min; 35 cycles of 30 s at 95°C, 45 s at 59.9°C and 60 s at 72°C; followed by 7 min at 72°C. The primer sequences, their positions and the PCR product sizes [15] are shown in *Table 2*.

Since the GenBank NCBI database is being constantly updated with new data, we checked the specificity of the primers recommended by the OIE for BLV diagnosis to make sure they are up to date. The primer verification and update was done using the Basic Local Alignment Search Tool (BLASTn) available at the National Center for Biotechnology Information (NCBI; Bethesda, MD). The multiple alignments of the nucleotide sequences and primers were performed with MUSCLE (Edgar, 2004) by MEGA5 software.

To optimize the nested-PCR, we ran consecutive gradient reactions. The annealing temperature range for the first primer pair (BLV-env-1 and 2) was 55.1°C - 66.2°C, and for the second one (BLV-env-3 and 4), 50.1°C - 66.3°C. The amplifications were run both with initial DNA template and with PCR products. The nested PCR temperature profile was as follows: Denaturation at 95°C for 3 min, 30 cycles, denaturation at 95°C for 30 s, annealing at 59.9°C or 61.1°C for 30 s, extension at 72°C for 50 s, final extension at 72°C for 7 min, storage at 4°C.

db Nested PCR

PCR was also performed without DNA extraction, directly on blood using a MyTaq Blood - PCR-Kit (Bioline, UK). The primers were the first primer pair that we used in the nested-PCR. The PCR program was optimized in terms of the step durations: denaturation for 15 s, annealing for 30 s, extension for 45 s, according to some requirements of the kit. The same PCR program was used with the internal primer pair. The amplification was run with 1, 2 and 3 µL of blood. The samples were re-assayed independently following storage at -20°C for 1 month.

The quality and quantity of the obtained DNA/PCR products were determined by DNA/RNA calculator GeneQuant II (Pharmacia LKB, Biochrom, UK) and by 2% agarose gel electrophoresis (Gene Shun Biotech, China) with a 100 bp DNA ladder (Bioline, UK). The electrophoresis conditions were: 120 V, 45 mA, 30 min.

Sequencing

To confirm the specificity of the products obtained after the first- and second-round PCR, 5 samples each were sequenced using a DYEnamic ET Dye Terminator Cycle Sequencing Kit (GE Healthcare, Giles, UK) in both directions with a forward and reverse primer. The obtained sequencing products were analyzed in a capillary MegaBACE™ 1000 automatic sequencer (Amersham Biosciences). Two controls, M13mp18DNA and MegaBACE 4 Colour Standart, of the kit used for the sequencing reaction and reading were included, respectively. The obtained sequences were analyzed by BLASTn (NCBI; Bethesda, MD).

Statistical Analysis

The agreement between the assays was evaluated using Cohen's kappa statistic according to McHugh [24], and the sensitivity and specificity of the assays were calculated according to Fenner et al.[25], ELISA was used as the gold standard [19].

Results

ELISA identified antibodies against BLV in 57 out of the 409 tested blood sera (13.9%). Of these, 27 showed doubtful/ inconclusive results in the first assay but proved negative following the confirmatory procedure in the ELISA kit.

Table 2. Primer sequences, position and product size used in the nested PCR for BLV detection			
Primers for *env* gp51 Gene	**Sequence (5'-3')**	**Position**	**Product Size, bp**
BLV-env-1	TS'TGTGCCAR'GTCTCCCAGATA	5032–5053	598
BLV-env-2	AACAACAACCTCTGGGAAGGG	5629–5608	-
BLV-env-3	CCCACAAGGGCGGCGCCGGTTT	5099–5121	444
BLV-env-4	GCGAGGCCGGGTCCAGAGCTGG	5542–5521	-
'updated positions			

Table 3. *Comparison between AGID and ELISA in BLV detection assays*

Tests Results	ELISA Positive	ELISA Negative	Total
AGID Positive	33	9	42
AGID Negative	24	343	367
Total	57	352	409

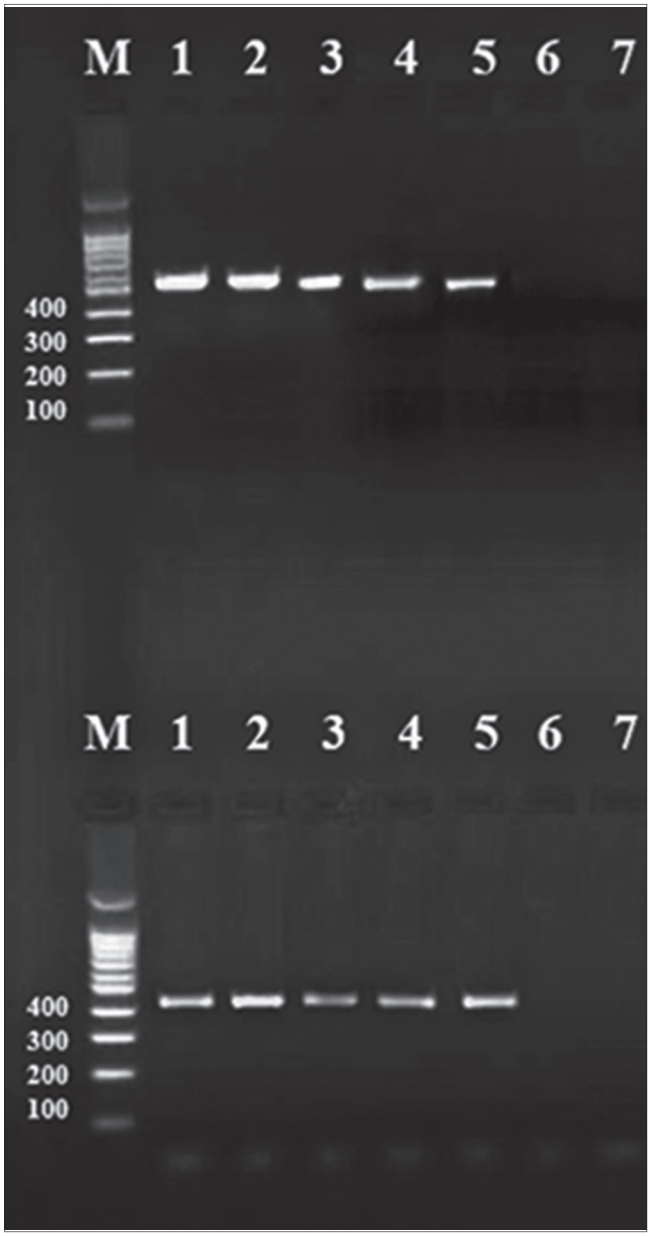

Fig 1. Results from nested and db nested PCR using two primer pairs, BLV-env-1 and 2 (top); BLV-env-3 and 4 (bottom); M - DNA Ladder 100 bp. Top, BLV-env-1 and 2: 1 - sample x; 2 - sample y; 3 - amplification of sample y directly from 1 µL blood; 4 - amplification of sample y directly from 2 µL blood; 5 - amplification of sample y directly from 3 µL blood; Bottom: 1 - sample x; 2 - sample y; 3 - amplification of sample y directly from 1 µL blood; 4 - amplification of sample y directly from 2 µL blood; 5 - amplification of sample y directly from 3 µL blood; 6 - negative sample by ELISA and AGID; 7 - negative control PCR

Table 4. *Comparison between nested PCR/db nested PCR and ELISA in BLV detection assays*

Tests Results	ELISA Positive	ELISA Negative	Total
Nested/db Nested PCR Positive	56	0	56
Nested/db Nested PCR Negative	1	352	353
Total	57	352	409

Table 5. *Data of kappa statistics showing agreement between AGID and ELISA and between nested PCR and ELISA*

Parameters	AGID/ELISA	Nested and db Nested PCR/ELISA
% Agreement	91.93	99.76
Kappa value	0.62	0.99
SE of kappa	0.06	0.01
95% CI	0.505 - 0.739	0.97 - 1.000

SE - standard error of kappa; CI - confidence interval

The results from the comparative analysis between AGID and ELISA are shown in *Table 3*. AGID identified 33/57 of the sera as positive, 9 of which as false positive. In addition, the results were inconsistent for a total of 22 sera in the first and the second testing. The sensitivity and specificity of the test were 57.9% (95% CI - 44.08 - 70.86) and 97.44% (95% CI - 95.20 - 98.82), respectively.

Gradient PCR on extracted DNA using the first primer pair had an optimum annealing temperature range of 59.7-62.6°C for amplification of a 598-bp product, and 57.0-64.3°C when a PCR product served as the template. The optimum annealing temperature range for the second primer pair was 58.9-61.1°C and 54.8-63.5°C, respectively, with a product size of 444 bp. The amplicons were best visualized using annealing temperature of 59.9°C for the BLV-env-1 and 2 primers, and 61.1°C for the BLV-env-3 and 4 primers, which we used in the subsequent analyses. The results from the nested and db nested PCR are shown in *Fig. 1*. Specific amplification was obtained both from extracted DNA and from whole blood, as well as using the three tested volumes of blood.

There was a second fragment about 100 bp in size in four of the positive samples. The sequencing procedure produced high background electropherograms, which did not allow BLASTn analysis to determine the origin of this fragment.

The sequencing results of the products from the first- and second-round PCR showed that products of about 538 bp and 397 bp, respectively, were suitable for analysis. BLASTn (NCBI) analysis confirmed that they belonged to BLV.

Table 4 presents the results from nested PCR and db nested PCR as compared to ELISA. There were no differences between the two PCR assays. As seen from *Table 4*, there

was inconsistency between nested PCR and db nested PCR vs. ELISA in only 1 sample, which tested negative by nested PCR/db nested PCR but positive by ELISA. The sensitivity of the nested PCR and db nested PCR was 98.25% (95% CI - 90.61 - 99.96%) and the specificity, 100.00% (95% CI - 98.96% - 100.00%).

The agreement between AGID and ELISA was 91.93%, with a kappa value of 0.62. The agreement of nested PCR and db nested PCR with ELISA was 99.76% and 0.99, respectively *(Table 5)*.

There were no differences between the results from the two independent tests run one month apart using either ELISA, or nested PCR and db nested PCR.

Discussion

The OIE Manual [15] recommends polymerase chain reaction, enzyme-linked immunosorbent assay and agar gel immunodiffusion test as suitable assays for the diagnosis of enzootic bovine leukosis. PCR detects sequences of the BLV *env* gene, whereas ELISA and AGID can detect antibodies against gp51, which is encoded by the *env* gene, and against the internal protein p24, which is encoded by the *gag* gene [26]. Antibodies against these proteins form shortly after infection onset and are detectable throughout the life of cattle [27]. In this study, we used four assays to test blood samples twice, the two tests being one month apart, to compare their effectiveness in the routine diagnosis of BLV.

A study has shown that AGID does not detect anti-gp51 antibodies in sera with low antibody titers associated with some physiological conditions in animals, such as advanced gestation or in the first days after calving, as well as antibodies in milk serum [28]. In our study, 42% of infected animals gave false negative results for the presence of antibodies in the AGID test. This was probably due to some of the above-mentioned factors in some of the animals. On the other hand, persistent co-infection with other viruses, such as bovine viral diarrhea virus, could lead to suppressed antibody formation against BLV [29]. Such cattle are an important source of infection from an epidemiological point of view. A drawback of AGID is the subjective factor in reading the results [30]. In our study, the results were interpreted independently by three experienced researchers, with no inconsistencies in the scoring. The second AGID testing produced inconsistent results in 22 sera: positive in the first test but negative in the second test following the freeze-thaw cycle. These are cases of weakly positive samples with faint lines of precipitation that remain undetectable possibly owing to partial antibody degradation in the second testing despite the low-temperature stability [31]. In addition, considering the type (size) of the antigen participating in the AGID reaction, which is a precipitation reaction in nature, the result will depend on the ratio between the antibody and antigen concentrations, forming a precipitation curve [32]. Besides, Rivers and Jones [33], who studied the titer of four types of IgG after 12 freeze–thaw cycles, reported that the titer of three types of IgG decreased two times after the first cycle. Based on these considerations, when a standard antigen concentration is used and the antibody concentration decreases as a result of a single freeze-thaw cycle of low-titer serum, consequently, the line of precipitation will shift to the left and visible complexes will not form. Such scenario could most likely explain the discrepancy between the results in the two AGID tests. This, along with the low sensitivity make AGID unsuitable in cases when there is irregular funding for EBL testing and samples need to be kept frozen until delivery of reagents.

Nine samples produced a false positive result, 4 of which in both tests, which was another surprising observation in this study. It could possibly be attributed to cross-reactive immune response in a natural infection with a genetically closely related retrovirus such as human T cell leukemia virus type I [34]. In addition, the k-statistic showed moderate agreement with ELISA with a kappa value of 0.62. Lojkić et al.[35] tested 12 AGID-negative sera and detected three positive samples using ELISA. Of 225 AGID-negative sera, Gonzalez et al.[36] found 69 ELISA-positive ones. Higher sensitivity of AGID than the 57.9% observed in our study was reported by Trono et al.[37]: 79.7% since AGID scored 36 out of 178 false-negative samples vs. PCR and southern blot analysis. In many countries, the costs of diagnostic tests for monitoring and elimination of EBL from farms are covered by the owners (incl. in the case of import and export of animals). Bulgaria is no exception. Thus, it is important to apply assays with high sensitivity and specificity to reduce the economic costs of farms [38] in the long run.

The high sensitivity of ELISA observed in this and other studies [26], the automated execution and interpretation of results and the use of minimum amounts of reagents make ELISA a very convenient screening method in the study of blood and milk sera to control the disease [39]. When compared to AGID, ELISA can detect anti-BLV antibodies earlier, from 3 to 12 weeks from infection onset [40], which is an advantage. Regardless of the ELISA kit used for antibody detection, Kuczewski et al.[41] reported that 5 kits produced by different companies showed high agreement between assays with kappa values of k=0.91 and k=1.

The procedures that we used for BLV detection by nested PCR and adapted db nested PCR generated amplicons of the expected size. The results showed that besides nested PCR, db nested PCR can also be used successfully as a key test in routine EBL diagnostics and as a confirmatory test for serological assays. In four of the samples, there was an additional amplified fragment which we were unable to identify after sequencing. Nevertheless, we could speculate that it might be attributed to activation of an endogenous retrovirus as a result of BLV proviral DNA integration, DNA

breakage during extraction [42] or a fragment resulting from *env* gene transcription. We could exclude the possibility of it being a fragment resulting from PCR because the same band appeared with different DNA concentrations.

In our study, there was just one sample that tested negative by nested PCR and db nested PCR but positive by ELISA. Similar results have been reported by Gregory et al.[43], who tested blood samples from cattle and interpreted 36/40 samples as positive using nested PCR, whereas 37/40 ones using ELISA. In another study, Villalobos-Cortés et al.[44] also observed ELISA to be more sensitive than nested PCR by 13%. Such discrepancy could possibly be explained by low virus titer in the infected animal, which remains below the detection threshold of nested PCR. The presence of proviral DNA and the low percentage of virus-infected cells, however, lead to constant stimulation of the immune system, which responds by producing antibodies. These antibodies - albeit present in a low amount - are detectable by ELISA [30,35]. Another reason could be a strong cytotoxic and humoral response in the first 1-8 weeks of viral infection [45], together with subsequent clearance and transition into latency via proviral integration. During latency, just 1:50 000 peripheral blood cells contain enough viral transcripts for them to be detected by *in situ* hybridization, a method of comparable sensitivity to PCR [46]. On the other hand, it is possible for not all peripheral blood mononuclear cells to be infected with the virus [27,47]. The samples that produced doubtful/inconclusive results in ELISA proved negative in the subsequent confirmatory assay, which entailed additional costs and time consumption, and was fully in agreement with the results from nested/db nested PCR. The diagnostic abilities of the three assays in our study were good, as evidenced by the kappa value of 0.99, which is interpreted as nearly perfect agreement according to McHugh [24]. A study reported 100% agreement between PCR and ELISA results [48]. Conventional PCR and INTA-ELISA showed over 90% agreement [37]; and direct filter PCR and ELISA showed 97.6%, strong agreement [19] with a kappa value of 0.88. Other authors report higher sensitivity of PCR compared to ELISA and nested PCR [17,49], however, this concerns mainly real-time quantitative PCR analysis, which still finds limited application in the routine diagnosis of EBL. In case of doubtful/inconclusive ELISA results, and following the kit's confirmatory procedure, PCR is recommended as an arbitration technique for detection of BLV proviral DNA [15].

The higher sensitivity of the PCR assays in our study, as compared to other reports of lower sensitivity of PCR vs. ELISA, could possibly be attributed to the capability of detecting more virus variants following the update of the first primer pair. Regarding the diagnostic abilities of direct blood (db) PCR, without DNA extraction, Nishimori et al.[20] observed lower sensitivity compared to nested PCR, but 100% specificity and reproducibility of results in cattle blood samples. In our study, there was full agreement between the results obtained using nested PCR and db nested PCR. This could probably be due to the fact that we used the same primers. These results indicate that, regardless of whether DNA is extracted or not, these particular PCR procedures will produce equally reliable results.

In conclusion, the low AGID sensitivity of 57.9% in this study and the inconsistent results it produced for 22 samples in two independent tests suggest that the future use of this assay in the routine EBL diagnostics should be reconsidered. The choice of method depends on the testing purpose and the population size. In eradication programs, it would be inappropriate to use AGID. This analysis would also be unsuitable for screening purposes in small farms because of the high error rate. Its implementation may be justified, to a certain extent, in large farms for initial screening; however, a positive result and confirmation by ELISA would increase the costs. Although AGID has advantages in terms of speed, ease of performance and no need of specific equipment, other highly specific assays, such as PCR and ELISA, will be required for disease control and successful monitoring programs.

Availability of Data and Materials

Datasets analyzed during the present study are available in the authors on reasonable request.

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Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

NR, IS and MCh designed the study; IS and MCh performed the samples collection and laboratory assays; NR analysed the data; NR, IS and MCh wrote the paper..

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