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COMPARISON OF AGAR GEL IMMUNODIFFUSION TEST, ENZYME-LINKED IMMUNOSORBENT ASSAY AND PCR IN DIAGNOSTICS OF ENZOOTIC BOVINE LEUKOSIS*

POREĐENJE AGAR GEL IMUNODIFUZIONOG TESTA, ENZIM VEZANOG IMUNOSORBENT TESTA I PCR U DIJAGNOSTIKOVANJU ENZOOTSKE LEUKOZE GOVEDA

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Bovine leukaemia virus (BLV) is a retrovirus that induces a chronic infection in cattle. Once infected, cattle remain virus carriers for life and start to show an antibody response within a few weeks after infection. Eradication and control of the disease are based on early diagnostics and segregation of the carriers. The choice of a diagnostic method depends on the eradication programme, money resources and characteristics of the herd to be analysed. The agar gel immunodiffusion (AGID) test has been the serological test of choice for routine diagnosis of serum samples. Nevertheless, in more recent years, the enzyme-linked immunosorbent assay (ELISA) has replaced the AGID for large scale testing. For this purpose, commercially available BLV-ELISA kits were compared to the AGID and to the polymerase chain reaction (PCR) method performed with two sets of primers, amplifying env region. The ELISA kit based on the p24 core protein was found to be less specific and served as a screening test. The ELISA kit based on the envelope glycoprotein (gp51) served as a verification test and gave a good correlation with the AGID test and PCR method. However, ELISA showed a higher sensitivity than AGID. The p24 based ELISA was useful for screening a large number of samples, whereas gp51 based ELISA, AGID and PCR were more important for detecting the antibody response against the individual BLV-proteins and therefore for verification of the infection with BLV.

Key words: AGID, diagnostics, ELISA, Enzootic bovine leukosis, PCR

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Introduction / *Uvod*

Bovine leukaemia is a disease of cattle characterised by the development of malignant lymphomas (lymphosarcomas). In Europe and also in South Africa, the disease is better known as bovine leukosis. It is further divided into enzootic and sporadic bovine leukosis, which contributes to some confusion when people are not familiar with the different forms of the disease [14]. Enzootic bovine leukosis (EBL) is a disease of adult cattle caused by the retrovirus, the bovine leukaemia virus (BLV). Cattle may be infected at any age, including the embryonic stage [8]. Most infections are subclinical, but a proportion of cattle over 3 years of age develop persistent lymphocytosis, and a smaller proportion develop lymphosarcomas in various internal organs [19]. Natural infection has also been recorded in buffalos, sheep and capybaras. BLV-infected animals usually demonstrate a strong humoral response to BLV, which can be exploited for diagnostics by serological techniques [1, 6]. Eradication and control of the disease is based on early diagnostics and segregation of the carriers. The sensitivity of the testing strategy is a critical consideration, as false-negative test results may unnecessarily prolong the eradication efforts [21]. For a number of years, the AGID test has been the prescribed test for international trade [22]. In more recent years, the ELISA has replaced the AGID in eradication programmes [7]. Nowadays, sequence data of different BLV proviruses are available, enabling the development of PCR that is increasingly used for the diagnostics of the EBL and has advantages over serological tests [2, 11, 12]. The aim of this study was to evaluate the practical application of PCR in parallel with routine by used serological methods, for detection of BLV, considering conditions with very low incidence of BLV infection.

Materials and methods / *Materijal i metode rada*

Samples / *Uzorci*

Serum samples were collected from dairy and beef herds from Slovenia. Only positive sera, as revealed with ELISA screening, were used in the further study. In BLV positive animals, samples were collected once more for sera and for DNA isolation.

AGID /*AGID*

AGID was performed in plastic Petri dishes (Ø 90 mm) filled with 15 ml of prewarmed liquid agar (Agar Mixture for Bovine Leucosis Immunodiffusion; Bommeli, Switzerland) to get a layer 2.5 mm thick. Cooled agar was punched to get a ring of wells. The centre well was filled with 25 μl of antigen (Bovine Leucosis Antigen for Immunodiffusion; Bommeli, Switzerland), and brim wells were filled with 50 μl of positive control serum (Bovine Leucosis Control Serum Positive, for

Immunodiffusion; Bommeli, Switzerland) or sample serum. We evaluated results after 72 hours of incubation at room temperature in a humid atmosphere.

ELISA / ELISA test

The commercially available ELISA kits were used according to the manufacturer's instructions. The microtiter plates were coated with antigen p24 in screening tests (LEUCOTEST; Bommeli, Switzerland) or with gP51 protein in confirmatory test (ELISA Bovine Leukosis Serum Verification; Pourquier, France). Positive and negative controls provided with the kits were run with each assay. After all the prescribed incubation and rinse steps, the plates were measured on a spectrophotometer (TECAN, Austria) and the results were interpreted as recommended by the manufacturer.

DNA isolation and PCR / Izolovanje DNK i PCR

Viral DNA was isolated from whole blood using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and/or from serum using QIAamp UltraSens Virus Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The *env*-nested PCR was performed with the external primers ENV1 (5'-TCT GTG CCA AGT CTC CCA GAT A-3') and ENV4 (5'-AAC AAC AAC CTC TGG GAA GGG T-3') and internal primers ENV2 (5'-CCC ACA AGG GCG GCG CCG GTT T-3') and ENV3 (5'-GCG AGG CCG GGT CCA GAG CTG G-3') [2]. The reaction mix for first amplification was performed with a 50 μl volume containing 20 μl template DNA, 0.5 μl of each primer, 4 μl dNTPs (10 mM each), 5 μl 10 x PCR buffer, 3 μl $MgCl_2$ (1.5 mM) and 0.25 μl Platinum Polymerase (Invitrogen, 5 U/μl). 3 μl of PCR product of the first amplification was used as a template for the second amplification. The amplification protocols previously described by Beier et al were used. PCR products were electrophoresed in a 2% agarose gel and visualised with ethidium bromide staining.

Results / *Rezultati ispitivanja*

To assess the ability of the AGID, confirmatory ELISA and PCR for the correct discrimination of positive and negative samples, the 30 positive sera as revealed by the screening ELISA were compared as well as 10 negative sera. The results demonstrate that all AGID-positive sera showed reactivity in confirmatory ELISA. Moreover, 9 of the AGID–negative sera showed reactivity in confirmatory ELISA. On the other hand, comparison of confirmatory ELISA and PCR resulted in 100% matching, with the exception of 2 samples where AGID and PCR were negative (Table 1). Other PCR negative samples were negative also in serological tests.

Table 1. *Distribution of AGID values, confirmatory ELISA values and PCR within 30 positive sera and 10 negative sera tested by screening ELISA* /
Tabela 1. Raspored AGID vrednosti, potvrđujućih ELIZA vrednosti i PCR kod 30 pozitivnih seruma i 10 negativnih seruma testiranih ELIZA skriningom

Sample / *Uzorak*	Screening *ELISA* / *ELISA test*	AGID / *AGID*	Confirmatory *ELISA* / *ELISA kao potvrda*	PCR *env* / *PCR env*
5714	pos	pos	pos	pos
5715	pos	pos	pos	pos
121/48	neg	neg	neg	-
121/49	pos	pos	pos	-
121/53	pos	pos	pos	-
121/90	neg	neg	neg	-
141/1	pos	pos	pos	pos
141/10	neg	neg	neg	neg
141/11	neg	neg	neg	neg
141/2	pos	pos	pos	pos
141/3	neg	neg	neg	neg
141/4	pos	neg	pos	neg
141/5	pos	pos	pos	pos
141/6	pos	pos	pos	pos
141/7	pos	pos	pos	pos
141/9	neg	neg	neg	neg
203/2	pos	pos	pos	pos
24/2	pos	pos	pos	pos
24/3	pos	pos	pos	pos
24/4	neg	neg	neg	neg
2533	pos	pos	pos	pos
31/1	pos	pos	pos	-
31/2	pos	pos	pos	-
31/3	pos	pos	pos	-
580/5	neg	neg	neg	-
609	pos	neg	pos	-
647	neg	neg	doubt.	-
653	pos	neg	pos	-
68/2	pos	neg	pos	-
695	pos	neg	pos	-
728/2	pos	neg	pos	-
728/3	pos	neg	pos	-
728/4	neg	neg	neg	-
729/1	pos	pos	pos	-
729/2	pos	pos	pos	-
729/3	pos	pos	pos	-
729/4	pos	pos	pos	-
729/5	pos	pos	pos	-
761	pos	neg	pos	-
821	pos	neg	pos	neg

Discussion / *Diskusija*

In view of the increasing economic impact of BLV infection on the cattle industry, the availability of a highly sensitive and specific assay for the identification of BLV-infected cattle is of critical importance. Ideally, the assay should be practical, inexpensive, and able to be adapted for large-scale use [16]. According to our results, AGID, ELISA and PCR methods are quite adequate for routine diagnostics. The AGID test has been the prescribed test for international trade for a number of years due to its high level of sensitivity and specificity. In recent years, many authors reported that the prevalence of BLV infection in the examined herds might have been underestimated, because the prevalence of the disease was based on data obtained by AGID, which unlike ELISA, is significantly less sensitive [23]. From this point of view, our results obtained from AGID showed as lower sensitivity in comparison with confirmatory ELISA, and the reading of the AGID test is more subjective [5, 9]. Nowadays, the detection of antibodies by ELISA has been widely used in veterinary diagnostic laboratories for serologic diagnosis of BLV infection. In general, ELISA is highly practical and relatively inexpensive, so it can be easily implemented for large-scale testing, which is usually needed in serological surveys and in control-and-eradication programmes [13]. Screening ELISA is applicable for the first step in making a diagnosis due to its good sensitivity and reduced specificity; it gives, as expected, more positive results. Using confirmatory ELISA, which is more specific, we retested these positive samples and reduced the level of positive results. Serologically confirmed positive results should be confirmed also with direct virus detection using molecular methods [17, 20]. The detection of viral sequences by PCR provides a precise and suitable method for the direct diagnosis of BLV infection, particularly as a confirmation test after serological testing in case of serologically doubtful results and when maternal antibodies still persist in the serum of the calves [18]. The reliability of the PCR method was demonstrated as well in our study where two different serological methods in comparison gave the same result as PCR. In view of this, when choosing a diagnostic test for BLV, it is essential to analyse the aim pursued. In high seroprevalence herds, high specific tests such as AGID and ELISA should be used, to ensure that animals considered as positive are truly positive, even at the risk of obtaining false-negative results [10, 15]. When running a large number of samples, direct virological methods would be better, but the price and the simplicity of serological methods make them more advisable [10]. On the other hand, in low-seroprevalence herds it is advisable to choose methods with high sensitivity, such as PCR, to ensure that all animals considered as negative are truly negative, even to asume the risk of encountering false-positive results [2]. This reference is derived from the fact that PCR methods are able to detect animals, which might test seronegative [4]. In this respect, serological tests and tests for direct viral detection must complement each other.

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SRPSKI

POREĐENJE AGAR GEL IMUNODIFUZIONOG TESTA, ENZIM VEZANOG IMUNOSORBENT TESTA I *PCR* U DIJAGNOSTIKOVANJU ENZOOTSKE LEUKOZE GOVEDA

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Virus leukemije goveda (*BLV*) je retrovirus koji ukazuje na hroničnu infekciju goveda. Kada se jednom zaraze, goveda ostaju nosioci virusa do kraja života i počinju da pokazuju odgovor antitela već nekoliko nedelja posle inficiranja. Iskorenjivanje i suzbijanje bolesti zasnovani su na ranoj dijagnostici i izolovanjem nosilaca. Izbor dijagnostičke metode zavisi od programa za iskorenjivanje bolesti, raspoloživih finansijskih sredstava, kao i od karakteristika stada koje treba da se analiziraju. Agar gel imunodifuzioni test (AGID) je već dugo odabrani serološki test za rutinsku dijagnozu serumskih uzoraka. Ipak, tokom poslednjih nekoliko godina enzim vezani imunosorbent test (*ELISA*) zamenio je AGID za testiranja velikog broja životinja. Usled toga, *BLV-ELISA* kitovi dostupni na tržištu upoređeni su sa AGID testom i metodom testiranja lančane reakcije polimeraze (*PCR*) koristeći dva seta prajmera, povećavajući *env* region. Utvrđeno je da je *ELISA* kit zasnovan na unutrašnjem proteinu p24 manje specifičan i da služi kao skrining test. *ELISA* kit zasnovan na *env* glikoproteinu (gp51) poslužio je kao verifikacioni test i dao je dobru korelaciju sa AGID testom i PCR metodom. Međutim, *ELISA* je pokazala veću osetljivost nego AGID. *ELISA* zasnovana na p24 bila je korisna za skrining većeg broja uzoraka, dok su *ELISA* zasnovana na gp51, AGID i PCR bili važniji za otkrivanje odgovora antitela prema pojedinačnim BLV-proteinima i prema tome, za verifikaciju *BLV* infekcije.

Ključne reči: AGID, dijagnostikovanje, *ELISA*, enzootska leukoza goveda, PCR

РУССКИЙ

СРАВНЕНИЕ АГАР ГЕЛЬ ИММУНОДИФФУЗИОННОГО ТЕСТА, ЭНЗИМ, СВЯЗЯННОГО ИММУНОСОРБЕНТ ТЕСТА И ЦРП В ДИАГНОСТИЦИРОВАНИИ ЭНЗООТИЧЕСКОГО ЛЕЙКОЗА КРУПНОГО РОГАТОГО СКОТА

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Вирус лейкемии крупного рогатого скота (ВЛКРС) ретровирус, указывающий на хроническую инфекцию крупного рогатого скота. Когда однажды заразится, крупный рогатый скот остаёт носитель вируса до конца жизни и начинает показывать ответ антител уже несколько недель после инфицирования. Искоренение и подавление болезни основанные на более ранней диагностике и изолированием носителей. Выбор диагностики метода от программы для искоренения болезни, наличные финансовые средства, словно и характеристик стада, которое

надо анализировать. Агар гель иммунодиффузионный тест (АГИД) уже долго отобранный серологический тест для умелого диагноза сывороточных образчиков. Всё-таки, в течение последних несколько лет энзим, связанный иммуносорбент тест (*ELISA*) заменил АГИД для тестирований большого числа животных. Вследствие того, *BLV-ELISA* киты доступные на рынке, сравненные с агид тестом и методом тестирования цепной реакции полимеразы (ЦРП), пользуя два сета праймера, увеличивая *env* регион. Утверждено, что *ELISA* кит основан на п24 внутреннем протеине меньше специфический и что служит как скрининг тест. ELISA кит основан на его гликопротеину (гп51) послужил как верификационный тест и дал хорошую корреляцию с АГИД тестом и ЦРП методом. Между тем, *ELISA* показала бóльшую чувствительность, чем АГИД. *ELISA* основана на п24 была полезная для скрининг бóльшего числа образчиков, пока *ELISA* основана на гп51, АГИД и ЦРП были более для открытия ответа антител к отдельным ВЛКРС-протеинами, и соглосно этому, для верификации ВЛКРС инфекции.

Ключевые слова: АГИД, диагостицирование, *ELISA*, энзоотический лейкоз крупного рогатого скота, ЦРП