results. The results of investigations indicate that it is necessary to exert control on the antibiotics residue in the milk after withdrawal period.

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ВИЯВЛЕННЯ АНТИБІОТИКІВ У МОЛОЦІ В ЗАЛЕЖНОСТІ ВІД СПОСОБУ ЗАСТОСУВАННЯ ПРЕПАРАТУ

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У статті наведені результати дослідження наявності залишку пеніциліну в молоці. Досліди проводили на коровах протягом 5 діб, використовуючи Charm MRL™ Test і SNAP Test після внутрішньом'язового та інтрамаммарного введення. У корів з інтрамаммарним застосуванням препарату виявлено виділення залишку з очищеної чверті та з інших трьох частин вимені. У результаті місцевого застосування виявлено, що концентрація залишку антибіотиків є вищою в молоці з очищеної чверті та значно нижчою в молоці з інших трьох чвертей. Внутрішньом'язове застосування пеніциліну протягом 48 годин призводить до його високої концентрації, яка згодом знижується. Порівнюючи результати двох тестів, було встановлено, що SNAP Test є більш чутливим і дає позитивні результати через 72 години, у той час як Charm MRL™ Test дає негативні результати.

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INFECTION WITH BOVINE LEUKEMIA VIRUS: CURRENT DIAGNOSTIC METHODS AND MOLECULAR EPDEMIOLOGY

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Bovine leukemia virus, a bovine retrovirus of worldwide distribution, is the etiological agent of enzootic bovine leucosis (EBL). The economic losses associated with EBL include the culling of infected cattle, reduced milk production, and trade restriction. In most of cattle-raising countries EBL is a notifiable disease, and official control measures include screening or monitoring and stamping out of infected animals. In this paper, we highlight the use of throughput screening assays (AGID and ELISA) and alternative methods (PCR) as well as give a new data related to the molecular epidemiology and genetic variability of BLV.

Introduction. Bovine leukemia virus (BLV), an exogenous retrovirus, is the causative agent of bovine lymphosarcoma, enzootic bovine leucosis. Pathogenic potential of BLV is clear; the virus infects preferentially and transforms B lymphocytes, but has also been found in T cells, monocytes and granulocytes. Infection by BLV may remain silent clinically as an aleukemic form but about one-third of infected cattle develops persistent lymphocytosis and 5 to 10% lymphoid tumours. Nowadays lymphosarcoma cases are rarely found in most European countries, however, BLV infections are currently found in some ones. Because of different status of level of eradication of BLV in EU Member States and neighbouring countries, control of BLV is important for the eradication of infection and for the national and international animal trade. There are two main motives for application of EBL eradication programme: to decrease the economical losses and improve food-producing animals'

health (3). BLV is not very pathogenic and infectious under natural conditions, however, economical losses are related to BLV-induced tumours, decrease in dairy production, early culling of animals and trade restrictions. According to the UE Directives (78/52 and 64/432) the tumoural form of bovine leukosis was declared as a 'legally contagious reputed disease' and this implies the systematic culling and removal of animals with tumoural lesions and screening for seropositive animals. Each EU Member State shall ensure that eradication programme will be established and the competent authority (veterinary inspection) will take appropriate measures to prevent any spread of the disease. In addition, in the recent years increasing importance has been attached to the conception of food quality. Therefore, the consumer might react against the presence of retrovirus in meat and milk of infected cows. In this paper methods, currently used for BLV detection and methods used in molecular epidemiology of BLV have been reviewed.

Serological methods. All BLV eradication programmes are based on the serologic tests. Since 1969, when BLV has been recognized, varieties of serological tests were used. However, according to the OIE Manual the agar gel immunodiffusin test (AGID) and enzyme-linked immunosorbent assay (ELISA) are the official reference (prescribed) tests only for the identification of BLV infection. Because ELISA tests are available in many different formats (competition, monoblocking, double-well), a recommendation was made to standardize assays using a known reference sera. These international standard reference sera, E1 and E5, define the lower limit of sensitivity of both AGID and ELISA for serum as well as milk. Annulal ring test should be performed by OIE and national reference laboratories on panel of sera from infected and uninfected animals to facilitate quality assurance and to ensure agreement between the different assays applied. AGID test is highly specific, technically simple and not expensive, however, its disadvantages are: low sensitivity (Abs detected after 4–8 weeks a.i.), reading results possible after 3 days and interpretation of results is highly subjective. ELISA is highly sensitiv (Abs detected after 2–4 weeks a.i. with good specificity). Test can be performed in a few hours and it is very suitable for large screening. Occasionally false positive results can occur. When using for routine testing, when the prevalence is high, the AGID seems to be a reliable indicator of BLV infection. This test is unable, however, to detect low level of antibodies shortly after infection and generally is less sensitive that ELISA. In opinion of many authorities, the AGID is not sensitive enough for classifying animals as BLV free and there is strong urge that the application of this test as «prescribed test» for international trade should be reconsidered. Since 2000, the predominant method used in Poland was ELISA with pooled serum samples. Commercial ELISA kits were obtained from IDEXX (USA) and Institute Pourquier (France) and the sensitivity and specificity of these kits were 99.6 and 99.7 %, respectively. While in 1999 out of 1373259 tested cattle 21697 (1.58 %) were seropositive, in 2008 only 5244 (0.38 %) out of 1365683 were detected.

PCR methods. The general use of serological methods is hampered frequently by the fact that BLV infected cattle can be found with low, transient or without BLV-antibody titers. It is therefore important to determine the infection status by direct detection of BLV. Since the virus remains latent *in vivo*, its direct detection is difficult for routine diagnosis. It has been shown that detection of proviral DNA of BLV by the polymerase chain reaction (PCR) provides a very sensitive means of direct diagnosis. The majority of the PCR assays are based on a single PCR, however, it was shown that in nested PCR less than eight genome copies of the provirus could be detected in the background of two million normal lymphocytes. Although PCR is not designed to replace traditional antibody assays, it can provide a useful tool for the early detection of BLV and for confirming BLV infection when weak positive or uncertain results are generated by ELISA. Recommendation for the PCR are: weak positive or doubt results in ELISA, in tumour cases during meet examination for differentiation between sporadic and BLV-induced lymphoma, in young calves with colostral antibodies for more active testing of calves under eradication programme.

Modification called PCR *in situ* is a method allowing the detection minute quantities of DNA or RNA directly in the intact cells or tissue sections, without necessity of DNA extraction. Another advantage over conventional PCR is that *in situ* PCR amplification signal is localised in specific cells or tissues. We developed *in situ* PCR for the detection

of proviral DNA of BLV in paraffin-embedded tissues and tissue smears of experimentally inoculated sheep as well as in peripheral blood leukocytes of naturally infected cattle. Ten sheep, five months old, were experimentally infected with BLV by intramuscular inoculation of 1×10^6 peripheral blood leukocytes of BLV-infected cow liver, spleen, lung, heart, kidney, and lymph node. Tissue smears were made using small pieces of freshly collected organs by simple printing them on the slides. The replicas of the tissues were air dried and fixed for 15 min in ice-cold 40 % acetone in PBS. Then the slides were again air dried and stored at – 20 °C until use. 5×10^4 peripheral blood leukocytes were seeded on silanised glasses by centrifugation for 15 min at 1 500 rpm, air dried, and fixed as tissue smears. The tissues were fixed for at least 14 h in 10 % buffered formalin, dehydrated in an increasing gradient of ethanol, paraffin embedded, and cut on a microtome to 5 μm thick sections. The sections were deparaffinised in xylene, rehydrated in a decreasing gradient of ethanol, washed twice in a sterile ddH_2O, and air-dried. By *in situ* PCR each specimen on a slide was covered with the adhesive frame. To made in this manner chamber, 65 μl of the reaction mixture, containing 10 mM Tris-HCl, pH 8.8, at 25 °C, 50 mM KCl, 2 mM $MgCl_2$, 0.1 % Triton X-100, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 190 μM dTTP, 10 μM DIG-11-dUTP, 2 U of DyNAzyme II polymerase, 0.2 μM of each env 5812R (ZM2) and env 5473F (ZM3) primers, was added. The chambers were covered with a polyester cover slip and the slides were placed on the *in situ* block of a thermal cycler. The reaction conditions were as follows: 95 °C for 5 min, 35 cycles of 94 °C for 1 min, 68 °C for 1 min, 72 °C for 1 min and a final elongation step for 7 min at 72 °C. After the amplification, the frames were removed and the slides were covered with the Coverplate Disposable Immunostaining Chamber and transferred to an appropriate stand. The slides were washed two times with 2 ml of TBST (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 % Tween 20). Next, to each chamber 2 ml of 1 % H_2O_2 in methanol was added and the slides were incubated for 30 min at 37 °C. After incubation, the slides were washed three times with 2 ml of TBST and to each chamber 250 μl of 1:50 diluted in TBS with 1 % blocking reagent anti-DIG-peroxidase conjugate was added and incubated for 1 h at 37°C. The slides were washed three times with 2 ml of TBST and to each chamber 1 ml of substrate was added (1.3 mM DAB in TBS with 0.02 % H_2O_2 and 0.03% $NiCl_2$ – for the specimens from paraffin embedded tissues and peripheral blood leukocytes and 1mM AEC in 50 mM acetate buffer with 0.015% H_2O_2 – for the tissue smears) and incubated for 30 min at 37°C. Next the slides were washed three times with 2 ml of TBST and then with ddH_2O. **Conclusion:** all tested samples, i.e. tissue sections, tissue smears, and peripheral blood leukocytes, were positive for BLV by *in situ* PCR. In general, BLV-positive signals were located more frequently in leukocytes infiltrating examined tissues than in other cell types. Therefore, *in situ* PCR may be used as a supportive or confirmatory method for differentiating between EBL and other lymphosarcomas of unknown etiology.

Molecular epidemiology of BLV. Genetic variation in the *Deltaretrovirus* genus appears to be minimal as compared with that of the other groups of *Retroviridae* family, represented mainly by lentiviruses. Nevertheless, the study of genetic diversity of BLV is important because it allows monitoring for the appearence of unusual viral variants and subtyping of the virus for epidemiologic studies as well as provides the phylogenetic information on the origin of the virus and tracing the movements of cattle. Initially, nucleotide sequence polymorphism was detected as restriction fragment length polymorphism (RFLP) (1). RFLP analysis revealed the presence of specific restriction sites through the genome that allows to classify BLV in European, Australian and North-American-Japanese subgroups. This was confirmed by sequence analysis of three isolates from Belgium, Japan and Australia that revealed 3% differences, mainly in point mutations. Although RFLP is very useful for distinguishing different restriction fragments, they can be detected only when DNA polymorphisms correspond to specific sites recognized by restriction endonucleases. Therefore, RFLP method may not be sufficiently discriminatory for epidemiologic study, but the single-strand conformational polymorphism (SSCP) provides a method to assess genetic variability among related viral strains.

We analysed BLV variants by both methods. Two hundred and one Holstein cows, 4–8 years old, from eight herds with a high prevalence of BLV infection were used for this experiment. The animals were selected on the basis of specific antibodies detected by ELISA and AGID tests (Synbiotics) as well as integrated proviral DNA, evidenced by PCR. The sequences of primers used in nested PCR were selected from a conserved part of the *env* gene. The sequence of forward primers were: 5'-TCTGTGCCAAGTCTCCCAGATA-3' and 5'-CC CACA AGGC GGCGCCGGTTT-3'; the sequence of reverse primers were 5'-GCGAGGCCG GGTCCAGA GCTGG-3' and 5'-AACAACAACCTCTGGG AAGGGT-5'. In each 50 μl reaction, 0.2 mM of each dNTPs (Amersham), 25 pmol of primers, 5 μl of *Taq* polymerase (10X) buffer, 2.0 U of *Taq* polymerase (Finzymes) and 1 μg of DNA template were used. Thermal cycling was carried out using a PEC Thermal Cycler 480 as follows: 94 °C for 1 min, 62 °C for 1 min, 72 °C for 2 min with final extension at 72 °C for 10 min. Cycling conditions for the second round were the same except 68 °C for the annealing step. Nested-PCR resulting in the amplification of a 444-bp fragment was done with 3 μl of first PCR amplified product. In SSCP analysis ten microliters of the PCR-amplified DNA fragments were diluted 1:2 in a denaturing loading mix (95 % formamide; 0.05 % bromophenol blue; 0.05 % xylene cyanol) and heated at 95 °C for 5 min and then chilled on ice for at least 5 min. Fifteen microliters of each diluted sample were loaded onto a non-denaturing 8 % polyacrylamide gel containing 8 % glycerol. Electrophoresis was performed using a Protean II apparatus (BioRad). Samples were run in TBE (53 mM Tris-HCl; 53 mM boric acid; 1.5 mM $EDTA_2Na$) buffer at 25 mA for 6 h at 6 °C. After electrophoresis, the gels were stained in 0.5 μg/ml ethidium bromide for 10 min, and then analysed under UV light using a Gel-Doc 1000 System (BioRad). In RFLP direct digestion of 10 μl of nested-PCR amplified product with 5 U of *Bcl*I, *Pvu*II or *Bam*HI endonuclease (Boehringer, Mannheim) was used to verify the specificity of amplicons and to determine the subgroup of BLV. **Conclusion:** 78 cattle were identified as positive by amplification of 444 bp *env* fragment of BLV using the nested PCR. 72 PCR-positive samples were subjected to RFLP analysis, and showed the prevalence of four different restriction types. The restriction pattern corresponding to the Belgian subtype was observed in 48 (66.6 %) samples, the pattern of Australian subtype in 8 (11.1 %) and the Japanese subtype in 7 (9.7 %) samples. Nine samples (9.7 %) represented unknown restriction pattern. All 48 BLV samples representing Belgian subtype were first analysed to determine whether SSCP enabled to differentiate BLV samples of the same restriction. The results showed that 14 specimens of Belgian type represent distinct migration patterns. Analysis of these samples can distinguish seven migration patterns, suggesting the presence of different proviral sequences within samples of the same restriction type. This confirms the capability of SSCP to detect minor sequence differences and to discriminate BLV variants that were indistinguishable by RFLP analysis. In fact, SSCP can be useful as an easy tool in epidemiological approach when different BLV provirus variants in one herd may result from implementation of BLV-infected cattle from other herds.

Genetic diversity of *env* gene of BLV. Infection with BLV induces strong humoral response directed mainly to the gp-51 envelope glycoprotein. Detailed analysis of *env* gene region encoding for gp-51 protein revealed the presence of three epitops: F, G (G_1,G_2) and H which represent major immunodominants on the envelope glycoprotein. Sequence variations of the BLV *env* gene appears to be minimal as compared to several other lentiviruses, however, certain point mutations and substitutions of amino acids arising in the major immunodominants of gp-51 may give rise to antigenic variations. The existence of *env* gene mutants with diminished antigenicity has been reported (2). Because serological tests, based on detection of antibodies to gp-51 protein, are largely used in the control of BLV infection, the presence of genetic variants of BLV can circumvent the immune response and in fact influence the effectiveness of serological methods. In our study, we determined the nature of genetic heterogeneity of the *env* gene of BLV in the context of its influence on serological response. Proviral DNA-BLV from 116 seronegative cows, selected out from 201 dairy cattle of nine herds, was amplified by nested-PCR using primers spanned 3' end

of *env* gene encoding F, G and H epitopes. Serological examination was performed by the use of ELISA test (Synbiotics, France). A 286 bp fragment of proviral DNA was sequenced and analysed by single-strand conformational polymorphism (SSCP). For sequencing PCR products were ligated to the pGEM-T Easy plasmid vector (Promega) by TA cloning and six plasmid clones for each PCR sample were sequenced and a consensus sequence was generated. Resulted sequences were aligned to prototype strain of BLV (FLK isolate, GeneBank Acc, number M35238), analysed by the MegAlin 3.1.7. programme from the DNASTAR package (DNASTAR Inc.) and subsequently with the Clustal X. The GeneDoc 2.1.00 editor programme was applied to prepare the final version of alignment with the consensus sequences. A bootstrapped consensus phylogenetic three was constructed using the neighbour-joining method with Clustal X and plotted by means of the Njplot programme. The most of changes were point mutations concerned epitopes G and H, while no changes were found within epitope F. In all 15 isolates G_1 epitope was modified (GCA-ACA) leading to the amino acid (aa) substitution (Ala-Thr). Sequences of five isolates (subgroup A) were characterized by the lacking epitope H (TCC-GCC) and G_2 (GCC/AAG-CCC/AGG) leading to substitution Ser-Ala and AlaLys-ProArg. In the next four isolates, creating subgroup B, epitope H was preserved, while epitope G_2 was modified by the following substitutions: GCC/AAG-CCC/AGA (AlaLys-ProArg). The remaining five isolates (subgroup C) lacking epitope G_2 (GCC/AAG-CCC/AGG) were characterized by additional point mutations distributed randomly between epitope G_1 and F. Phylogenetic three representing all isolates has partitioned all *env* sequences into three distinct clusters and there was strong support for the assembly of sequences into groups classified previously according to changes within particular epitopes. SSCP analysis performed with samples which sequences were closely related, as determined by pylogenetic analysis, revealed similar migration patterns. In all isolates, the similar band profiles were related to sequence identity. **Conclusion:** the results showed that genetic variants of BLV with certain point mutations within sequences encoding gp-51 epitopes were found in 19% of cattle naturally infected with BLV, but serologically negative. SSCP analysis was successfully used to distinguish these variants in the particular animals and in fact, can be useful as an easy tool in epidemiological study. In some animals, these nucleotide changes leaded to amino acid substitutions. We assume that resulted antigenic variants can escape antibody detection and would disturb the successful application of serological assay.

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ИНФИЦИРОВАНИЕ ВИРУСОМ ЛЕЙКЕМИИ КРУПНОГО РОГАТОГО СКОТА: СОВРЕМЕННЫЕ МЕТОДЫ ДИАГНОСТИКИ И МОЛЕКУЛЯРНАЯ ЭПИДЕМИОЛОГИЯ

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Вирус лейкемии крупного рогатого скота, ретровирус крупного рогатого скота широкого распространения, является этиологическим возбудителем энзоотичного лейкоза крупного рогатого скота. Экономические убытки, связанные с этим заболеванием, включают отбраковку инфицированных животных, снижение производства молока и торговые ограничения. В большинстве стран с развитым скотоводством лейкоз крупного рогатого скота является заболеванием, подлежащим регистрации, и официальные меры контроля включают массовое обследование или мониторинг и забой инфицированных животных. В этой статье мы осветили вопрос об использовании методов иммунодиффузии в агарозном геле, иммуноферментного анализа и альтернативных методов (ПЦР), а также представили информацию о молекулярной эпидемиологии и генетической вариабельности вируса лейкоза крупного рогатого скота.