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(54) Title: A STRIP TEST FOR DETECTING ENZOOTIC BOVINE LEUKOSIS AND A METHOD FOR DETECTING ENZOOTIC BOVINE LEUKOSIS WITH THE USE OF THE STRIP TEST

(57) Abstract: The object of the invention is a strip test for detecting enzootic bovine leukosis by assaying anti-BLV antibodies in a sample, characterized in that it contains the gp51 antigen obtained in a baculovirus system and immobilized on a nitrocellulose membrane and A, G, A/G proteins labelled with coloured nanoparticles, as well as glass fibre filter paper for filtering a sample, wherein the analysed sample is cow's milk. A further object of the invention is a method for detecting enzootic bovine leukosis with the use of the strip test according to the invention, comprising the following steps: a. A milk sample is diluted three times with 20 mm Tris-HCl buffer pH 7.5 containing 0.3 % casein and 0.15%Tween 20; b. The milk sample is instilled into the window on the test cassette and incubated for 10 minutes; c. A test result is read in the test window, one bar or spot indicating a negative result and two bars or spots indicating a positive result.

# A strip test for detecting enzootic bovine leukosis and a method for detecting enzootic bovine leukosis with the use of the strip test

The object of the invention is a strip test for detecting enzootic bovine leukosis by assaying anti-BLV antibodies in a cow's milk sample. The invention also relates to a method for detecting enzootic bovine leukosis with the use of this strip test.

Strip tests have been successfully used for many years in both medical and veterinary diagnostics, as well as in agriculture, the food industry and environmental protection. They are characterized by a simple design, high sensitivity and a low price. In veterinary medical practice, tests are used to test both farm animals (cows, poultry, sheep, pigs) and pets, mainly dogs and cats. Tests are used to detect bacteria, viruses, allergic diseases, but also to check, for example, reproductive ability.

Enzootic bovine leukosis (EBL) is an infectious disease in cattle caused by a C-type retrovirus called Bovine Leukemia Virus (BLV), and consisting in lymphatic system cell hyperplasia. The disease is characterized by a long incubation period (up to 7 years) and in most cases (approx. 60%) is asymptomatic, but this is not related to virus latency since some cells have been shown to express the virus (Powers et al., 1992). Some adult cattle (10-30%) develop lymphoceles, 1-10% develop lymphatic sarcomas on various internal organs with a marked increase in the number of B lymphocytes near the sarcoma. The most common symptoms are weight loss, decreased milking, digestive disorders, enlarged lymph nodes, posterior paralysis (Polat et al., 2017).

Enzootic bovine leukosis is one of the causes of significant financial losses in dairy herds. Studies show a decrease in the milk yield of BLV-infected cows from 2.5 to 2.7% and a 7% decrease in the number of conceptions (Ott et al., 2003).

Since the disease is asymptomatic for an initial, long period of time, the only effective way to prevent the spread of infection is frequent serological testing and elimination of infected animals. Serological tests are carried out in animals over 6 months of age when maternally derived antibodies disappear. The virus itself can be detected with the use of an electron microscope in isolated peripheral blood lymphocytes and viral DNA can be assayed by means of PCR method. To diagnose bovine leukosis, immunological methods are most commonly used: AGID (agar gel immunodiffusion assay), ELISA (enzyme-linked immunosorbent assay) and radioimmunoassays. These methods are employed to detect antibodies against gp51 and p24 antigen proteins. There is a lot of contradictory information in the literature as regards the usefulness of both antigens in diagnostics. However, it seems that antibodies against gp51 appear more rapidly in the serum, their titre is higher and they are present in positive samples in a much larger amount (Choi et al., 2002, Bicka et al., 2001).

PL214884 describes an ELISA test for detecting enzootic bovine leukosis characterized in that it contains a highly purified gp51 BLV antigen obtained from a completely bovine serum-free cell culture medium.

In turn, PL217257 discloses a method for detecting enzootic bovine leukosis and an ELISA detection kit characterized in that the presence of antibodies against antigen BLV proteins, in particular gp51 or p24 antigens, is detected in a sample collected from a tested animal, in particular in its serum or milk.

Non-patent literature describes an immunochromatographic strip test using colloidal gold-labelled gp51 antigen and a monoclonal antibody immobilized on a test membrane. The test is based

on competitive binding of gp51 antigen to bovine serum antibodies and monoclonal antibodies on the membrane. The test sensitivity is low, i.e. 1:10 as compared to the E05 reference serum (Kim et al. 2016).

Chinese application No. CN101303351 describes a strip test for detecting enzootic bovine leukosis in blood serum using gp51 protein and colloidal gold-based immunochromatography, the p51 antigen being expressed in a bacterial system and thus not being a glycoprotein.

Therefore, the object of the invention was to develop a simple and rapid test that could be performed under field conditions even by unskilled individuals, e.g. stock breeders.

The object of the invention is a strip test for detecting enzootic bovine leukosis by assaying anti-BLV antibodies in a sample, characterized in that it contains the gp51 antigen obtained in a baculovirus system and immobilized on a nitrocellulose membrane and A, G, A/G proteins labelled with coloured nanoparticles, as well as glass fibre filter paper for filtering a sample, wherein the analysed sample is cow's milk.

Preferably, the glass fibre filter paper is Whatman GF, MF1, AH934 or Fusion 5 glass fibre filter paper saturated with a solution of 20 mM Tris pH 7.5 with 0.2% casein and 0.1% Tween 20 added.

The strip test of this invention is characterized in that it contains the gp51 antigen optionally obtained in a non-baculovirus system.

The strip test of this invention is characterized in that the gp51 antigen is obtained in FLK-BLV cells.

Preferably, coloured nanoparticles are colloidal gold nanoparticles with a diameter of 20-50 nm, carbon nanoparticles with a diameter of 10-20 nm, or coloured polystyrene nanoparticles with a diameter of 50-200 nm.

A further object of the invention is a method for detecting enzootic bovine leukosis with the use of the strip test of this invention, comprising the following steps:

a. A milk sample is diluted three times with 20 mm Tris-HCl buffer pH 7.5 containing 0.3 % casein and 0.15% Tween 20;

b. The milk sample is instilled into the window on the test cassette and incubated for 10 minutes;

c. The test result is read in the test window, one bar or spot indicating a negative result and two bars or spots indicating a positive result.

The advantages of the developed test of this invention include:

a) use of a milk sample for assays (with the elimination of the stage of blood sampling and serum separation);

b) prevention of the spread of infections;

c) minimising of economic losses;

d) monitoring of milk purity for BLV infection.

The use of milk for the strip test makes the strip test difficult to carry out due to the presence of large amounts of fat. This problem has been solved by diluting milk with a solution containing detergents and using glass fibre filtering paper to filter fat on the test strip.

It is also significantly that even after 12 months the test retains about 83% of its original activity, which only slightly affects its sensitivity. The initial sensitivity is 1:1000, the final sensitivity is 1:800, and the required sensitivity for such a test is 1:250.

The test is characterized by high sensitivity and specificity and ensures good compatibility of the results with commercial ELISA tests.

| | ELISA test | |
|---|---|---|
| | positive | negative |
| | 88×33 | 88.58 |
| positive | 28 | 2 |
| negative | 3 | 96 |
| Strip test ↓ | | |

Sensitivity 90.3% Specificity 98.5%

The test gives a negative result with sera positive for other bovine viruses: BIV, BVD and BVH.

The object of the invention is illustrated in a figure showing a photograph of the developed strip test and its results with standard anti-BLV E05 serum diluted in normal milk.

The solution of this invention is presented in embodiments, which however, are not intended to limit the scope of the invention but rather to illustrate it in order to facilitate understanding.

**Examples**

**Example 1. Preparation of gp51 antigen in a baculovirus system**

Mimic Sf9 cells (Life Technologies, USA) were infected with a baculovirus with a subcloned gene encoding the gp51 protein. The virus titre was determined by a serial dilution method. The baculovirus construct was expressed in 1000 ml in a serum-free medium (CCM3, Becton-Dickinson or ESF921, Expression Systems). 48 hours following the infection with the baculovirus, the cells were centrifuged and the post-culture medium was dialysed for several dozen hours against phosphate buffer. The preparation was applied to an NiNTA-agarose bed and the bound protein was eluted with a step gradient of imidazole. The analysis of collected fractions was performed by dot-blot methods with the 9E10 antibody. The fractions with the highest gp51 concentration were combined (fractions eluted with 50, 100 and 200 mM imidazole) and dialysed against TBS buffer. The resulting preparations were then concentrated using Amicon cartridges (Merck Millipore, Germany). The purification degree of the preparations was tested by SDS-PAGE electrophoresis and CBB staining. The preparations eluted with 100 and 200 mM imidazole were characterized by the highest purification degree.

**Example 2. Preparation of colloidal gold-labelled A/G protein**

To 10 ml of 0,1% solution of colloidal gold with a particle diameter of about 40 nm, containing 10 mM borate buffer pH 8.0, 100 µg A/G protein was added and stirred for 1 h at room temperature. Then 1 ml of a solution containing 1% casein and 0.1% Tween 20 in 20 mM borate buffer pH 8.0 was added. The solution was centrifuged at 20,000 x g for 15 minutes, washed and suspended in 2 ml of 0.2 % casein with 0.05% Tween20 in 20 mm borate buffer pH 8.0.

**Example 3. Preparation of protein A labelled with carbon nanoparticles**

To 10 ml of 0.2% solution of carbon nanoparticles (Orion's Special Black 4 and Printex30 carbon nanoparticles or Dimacolor's Carbon Black 510 and 610 carbon nanoparticles of around 20 nm in diameter) in 5 mM borate buffer pH 8.0, 50 µg A protein was added and stirred for 18 h at 4°C. Then 1 ml of a solution containing 1% casein and 0.1 % Tween 20 in 20 mM borate buffer pH 8.0 was added. The solution was centrifuged at 20,000 x g for 15 minutes, washed and suspended in 2 ml of 0.2 % casein with 0.05% Tween20 in 20 mm borate buffer pH 8.0.

**Example 4. Test strip preparation**

The labelled A/G protein was applied to Millipore's GFDX Glass Fiber Conjugate Pads glass fibre paper. A solution of gp51 antigen (test line) and bovine immunoglobulins (control line) were applied, as a line or spot, to Millipore's HF120 or HF135 nitrocellulose membrane. The paper with the coloured conjugate was glued at the start of the nitrocellulose paper, while the glass fibre paper for applying the sample was glued to the conjugate. At the end of the nitrocellulose paper, plain cellulose paper was placed. The test strip was placed in a special plastic cassette.

**Example 5. Strip test performance**

A milk sample was diluted three times with 20 mM Tris-HCl buffer pH 7.5 containing 0.3 % casein and 0.15% Tween 20. 200 µl of the sample was instilled into the window on the test cassette and left for 10 minutes. The appearance of one bar or spot in the test window indicates a negative result, while the appearance of two bars or spots indicates a positive result.

The tests developed should become a valuable diagnostic tool for monitoring the health status of cattle directly in the field and can contribute to improving the efficiency of cow breeding economy. The test may also be used to monitor the purity of milk and meat.

## Claims

1. A strip test for detecting enzootic bovine leukosis by assaying anti-BLV antibodies in a sample, characterized in that it contains the gp51 antigen obtained in a baculovirus system and immobilized on a nitrocellulose membrane and A, G, A/G proteins labelled with coloured nanoparticles, as well as glass fibre filter paper for filtering the sample, wherein the analysed sample is cow's milk.
2. The strip test according to claim 1, characterized in that the glass fibre filter paper is Whatman GF, MF1, AH934 or Fusion 5 glass fibre filter paper saturated with a solution of 20 mM Tris pH 7.5 with 0.2 % casein and 0.1% Tween 20 added.
3. The strip test according to claim 1, characterized in that it contains the gp51 antigen optionally obtained in a non-baculovirus system.
4. The strip test according to claim 3, characterized in that the gp51 antigen is obtained in FLK-BLV cells.
5. The strip test according to claim 1, characterized in that the coloured nanoparticles are colloidal gold nanoparticles with a diameter of 20-50 nm, carbon nanoparticles with a diameter of 10-20 nm, or coloured polystyrene nanoparticles with a diameter of 50-200 nm.
6. A method for detecting enzootic bovine leukosis with the use of the strip test as described in claims 1-5, comprising the following steps:
    a. A milk sample is diluted three times with 20 mm Tris-HCl buffer pH 7.5 containing 0.3 % casein and 0.15% Tween 20;
    b. The milk sample is instilled into the window on the test cassette and incubated for 10 minutes;
    c. The test result is read in the test window, one bar or spot indicating a negative result and two bars or spots indicating a positive result.

# AMENDED CLAIMS
received by the International Bureau on 07 January 2021 (07.01.2021)

1. A strip test for detecting enzootic bovine leukosis by assaying anti-BLV antibodies in a sample, characterized in that it contains the gp51 antigen obtained in a baculovirus system and immobilized on a nitrocellulose membrane and A, G, A/G proteins labelled with coloured nanoparticles, as well as glass fibre filter paper at the beginig of the test strip for filtering the sample, wherein the analysed sample is cow's milk.
2. The strip test according to claim 1, characterized in that the glass fibre filter paper is saturated with a solution of 20 mM Tris pH 7.5 with 0.2 % casein and 0.1% nonionic surfactant added.
3. The strip test according to claim 1, characterized in that the coloured nanoparticles are colloidal gold nanoparticles with a diameter of 20-50 nm, carbon nanoparticles with a diameter of 10-20 nm, or coloured polystyrene nanoparticles with a diameter of 50-200 nm.
4. A method for detecting enzootic bovine leukosis with the use of the strip test as described in claims 1-5, comprising the following steps:
    a. A milk sample is diluted three times with 20 mm Tris-HCl buffer pH 7.5 containing 0.3 % casein and 0.15% nonionic surfactant;
    b. The milk sample is instilled into the window on the test cassette and incubated for 10 minutes;
    c. The test result is read in the test window, one bar or spot indicating a negative result and two bars or spots indicating a positive result.

## STATEMENT UNDER ARTICLE 19(1)

Amendments to the submitted claims are intended to clarify the present solution.

Clarification of Claim 1 by introducing the placement of glass fiber paper at the beginning of the test strip the test strip, which indicates the inventive step of said invention by adaptation of the test strip for analysis directly from whole milk sample. The strip test described in D1 and other documents is designed to detect BLV in *blood serum*. In our application, we present a test for milk analysis, which facilitates and speeds up the test for BLV. Due to the presence of fat and other solids in milk, the use of it requires very specific handling. Therefore, a piece of glass paper saturated with an appropriate substances is used in the invention. There is also plain absorbent paper at the end of the test strip, but this has nothing common with the filter paper at the beginning of the test strip. In our opinion this is an inventive technical effect of our test which is constructed especially to work with raw whole milk. Other tests described in literature (also in literature cited in ISR) are dedicated to work with *blood serum*.

Moreover, according to D1 or D2, gp51 antigen is expressed in *E.coli*, such product is not glycosylated and as such, it is not useful for immunological tests. Antigen is supposed to be of natural origin from FLK-BLV cells or expressed in baculovirus system. Even partially glycosylated antigen expressed in *Saccharomyces cerevisiae* is inactive. In literature there is a lot of information about influence of glycosylation on immunoreactivity for example: M Legrain, D Portetelle, J Dumont, A Burny, F Hilger, *Biochemical and immunological characterization of the bovine leukemia virus (BLV) envelope glycoprotein (gp51) produced in Saccharomyces cerevisiae*. Gene 1989 Jul 15;79(2):227-37. doi: 10.1016/0378-1119(89)90205-9.

Claim 2 is clarified by determination of type of detergent without using its trade name, by changing Tween20 into nonionic surfactant and by deleting the Whatmann types of glass fibre filter paper.

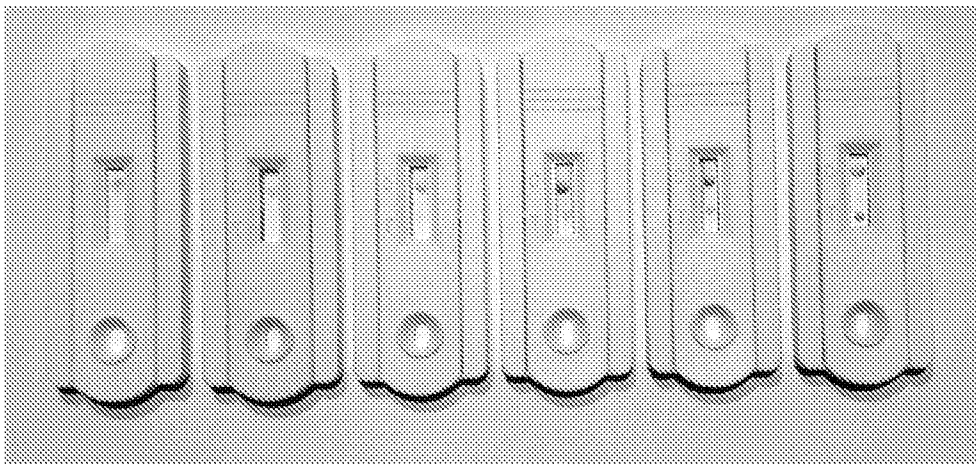
Claims 3 and 4 are cancelled.

Accordingly, Claim 5 is renumbered as new claim 3 – unchanged. It is worth to mention that in our solution in addition to colloidal gold nanoparticles (as disclosed in D2), the carbon or polymer nanoparticles may be used as well as indicates the new claim 3.

Claim 6 is renumbered as new claim 4 and in order to avoid the trade name, the term Tween 20 is changed to nonionic surfactant.

No new matter was added.

Fig.



1:3000 1:1000 1:300 1:100 1:30 1:10

# INTERNATIONAL SEARCH REPORT

International application No
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A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/569
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, COMPENDEX, EMBASE, FSTA, INSPEC, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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| Y | abstract<br>Materials and methods;<br>page 35, left-hand column, paragraph 1<br>figure 1<br>page 37, left-hand column, paragraph 3 | 1-6 |

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 28 October 2020 | 10/11/2020 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Routledge, Brian |

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Form PCT/ISA/210 (second sheet) (April 2005)

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# INTERNATIONAL SEARCH REPORT

International application No
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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