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DIAGNOSI S OF BOVINE LEUKOSIS IN IMPORTED DAIRY CATTLE IN KINGDOM OF SAUDI ARABIA USING THREE DIFFERENT TECHNIQUES

(With 4 Tables and 2 Figures)

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تشخيص مرض الليكوزس البقرى فى أبقار إنتاج الحليب المستوردة فى المملكة العربية السعودية بإستخدام ثلاث تقنيات مختلفة

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فى هذه الدراسة تم فحص عدد ٣٠٠ بقرة إنتاج حليب مستوردة من استراليا لتشخيص مرض الليكوزس البقرى من خلال التعرف على الأجسام المضادة والحامض النووى وأنتيجين فيروس اللوكيميا البقرى المسبب لهذا المرض وذلك بإجراء اختبار الإليزا (على عينات فردية من السيرم) والتفاعل التبلمرى المتسلسل (على عينات من الدم) وتقنية الصبغة المناعية النسيجية (على عينات من الغدة اللمفاوية فوق الضرع). مئتان من هذه الحيوانات تم فحصها بعد وصولها مباشرة إلى محجر ميناء جدة الإسلامى بالمملكة العربية السعودية أثناء فترة الحجر الإجباري (٢١يوم) وذلك بعمل الاختبارات السابق ذكرها مرة واحدة فقط. أما المائة بقرة المتبقية فقد تم اختيارها من مزرعة إنتاج حليب قامت باستيراد هذه الأبقار من استراليا أيضا قبل عام من بداية هذه الدراسة وتم فحصها بإجراء الاختبارات الثلاثة ثلاث مرات متتالية بفاصل زمنى شهر بين كل مرة و أخرى بالإضافة إلى فحص عينة حليب مختلطة تمثل كل المزرعة باستخدام اختبار الإليزا. خمسون من هذه المائة تم اختيارهم عشوائيا وخمسون تم اختيارهم من الأبقار ذات إنتاج الحليب المنخفض فى المزرعة. بالنسبة للأبقار حديثة الاستيراد الموجودة فى محجر الميناء , تم التعرف عل الأجسام المضادة لفيروس اللوكيميا فى عدد ١٦ بقرة والحامض النووى لفيروس اللوكيميا فى عدد٢١ بقرة و أنتيجين فيروس اللوكيميا فى عدد ٢٤ بقرة بعد إجراء اختبار الإليزا والتفاعل التبلمرى المتسلسل والصبغة المناعية النسيجية على التوالى. بعد عمل الفحص الأول لأبقار المزرعة تم التعرف على الأجسام المضادة لفيروس اللوكيميا فى عدد ٦ بقرات (خمسة منها من الأبقار ذات إنتاج الحليب المنخفض) والحامض النووى لفيروس اللوكيميا فى عدد ٧ بقرات (ستة منها من الأبقار ذات إنتاج الحليب المنخفض) وأنتيجين فيروس اللوكيميا فى عدد ٩ بقرات (ثمانية منها من الأبقار ذات إنتاج الحليب المنخفض). فى الفحص الثانى لأبقار المزرعة لم تتغير نتيجة اختبار الإليزا بينما زاد عدد الحالات الإيجابية إلى ٩ , ١١ مع التفاعل التبلمرى المتسلسل والصبغة المناعية النسيجية على التوالى. الفحص الثالث لأبقار المزرعة أظهر التحول السيرولوجى الذى حدث فى بقرتين أعطتا نتائج سلبية مع اختبار الإليزا الأول والثانى بينما نتائج التفاعل التبلمرى المتسلسل والصبغة المناعية النسيجية لم تتغيرا عن الفحص الثانى. عينة الحليب المشتركة الممثلة لكل المزرعة كانت

إيجابية للأجسام المضادة للفيروس بعد إجراء اختبار الإليزا عليها فى الثلاث فحوصات . نتائج هذا العمل أوضحت أن عدد غير قليل من أبقار إنتاج الحليب المستوردة من استراليا مصاب بمرض الليكوزس البقرى الذى ظهر من الدراسة أنه يؤثر على انتاج الحليب مما يؤكد على أهمية استخدام التشخيص المعملى لفحص أبقار إنتاج الحليب المستوردة قبل السماح بدخولها حيث كانت كل الحيوانات المصابة سليمة ظاهريا. أثبتت هذه الدراسة أن تقنية الصبغة المناعية النسيجية أشد حساسية من التفاعل التبلمرى المتسلسل واختبار الإليزا كما أن التفاعل التبلمرى المتسلسل أشد حساسية من اختبار الإليزا بالإضافة إلى أن تقنية المناعة النسيجية والتفاعل التبلمرى المتسلسل أمكنهما التعرف على الحالات الإيجابية الجديدة للإصابة بالفيروس قبل أن يتمكن اختبار الإليزا من ذلك. من كل النتائج التى تم تسجيلها فى هذه الدراسة يمكن التوصية بضرورة استخدام التفاعل التبلمرى المتسلسل مع اختبار الإليزا فى فحص جميع أبقار إنتاج الحليب المستوردة لتشخيص مرض الليكوزس البقرى وعدم السماح بدخول أى حيوان مصاب إلى داخل المملكة حيث لم يتم تسجيل هذا المرض رسميا بالمملكة حتى الآن بالرغم من أن العمل الحالى أثبت وجود هذا المرض فى أحد مزارع انتاج الحليب. للسيطرة على هذا المرض يجب فحص جميع مزارع انتاج الحليب خاصة تلك التى تعانى من نقص إنتاج الحليب دون وجود سبب واضح وذلك بعمل اختبار الإليزا على عينة حليب مختلطة تمثل كل المزرعة وفى حالة ايجابية هذه العينة يجب فحص جميع حيوانات المزرعة باستخدام اختبار الإليزا والتفاعل التبلمرى المتسلسل معا والتخلص من الحيوانات الإيجابية لهذا المرض. بالرغم من شدة حساسية تقنيةالصبغة المناعية النسيجية إلا أننى لا أوصى باستعمالها فى الوقت الحالى نظرا لتعقد خطواتها وتكلفتها العالية لكن مع استمرار العمل على تبسيط خطوات هذه التقنية وتقليل التكلفة قد تكون هى التقنية المثلى لتشخيص هذا المرض فى المستقبل.

SUMMARY

In this study, 300 dairy cattle imported from Australia were investigated for diagnosis of bovine leukosis through detection of bovine leukemia virus (BLV) antibodies, BL viral nucleic acid and antigen of BLV (which causes this disease) by performing, enzyme linked immunosorbent assay (ELISA) to serum samples, polymerase chain reaction (PCR) to blood samples and immunohistochemical stainig technique using avidine biotin peroxidase complex (ABC) method to lymph node biopsies respectively. 200 of these dairy cattle were investigated immediately After arrival to Islamic Jeddah Port, Kingdom of Saudi Arabia (KSA) during the obligatory quarantine period (21 days) by performing the three previously mentioned techniques one time. While, the remaining 100 dairy cattle were selected from one dairy farm imported these cattle from Australia also one year before beginning of the study and investigated by these techniques 3 successive times one month apart, in addition to investigate one pooled bulk tank milk sample by ELISA. 50 out of these 100 cattle were selected randomly and 50 selected from low producing cattle in the farm. Concerning to recently imported dairy cattle in port `s quarantine, BLV antibodies were detected in

16 cattle, BL viral nucleic acid was detected in 21cattle and BL viral antigen was detected in 24 cattle after performing ELISA, PCR and ABC respectively. After performing first investigation tom cattle of dairy farm, BLV antibodies were identified in 6 cattle (5 of them from low milk producer cattle), BL viral nucleic acid was detected in 7 cattle (6 of them from low milk producer cattle) and BL viral antigen in 9 cattle (8 of them from low milk producer cattle). In the second investigation of cattle of the dairy farm, no change in results of ELISA was observed while number of positive cases increased to 9 and 11 with PCR and ABC respectively. The third investigation of dairy farm cattle revealed seroconversion of 2 cattle that were negative with first and second ELISA while no change of PCR and ABC results were recorded. Pooled bulk tank milk sample was positive to BLV antibodies with 3 successive ELISA. Results of this work showed that not few numbers of imported dairy cattle were infected with bovine leukosis which seemed to has a correlation with low milk production. All infected cattle were apparently healthy, therefore, laboratory investigation of imported dairy cattle for BLV before introducing of these animals is very important. This study proved that ABC method is more sensitive than PCR and ELISA and PCR is more sensitive than ELISA, at the same time ABC and PCR could detect new infections before ELISA. All results of this study cleared the importance of performing ELISA and PCR to investigate all imported dairy cattle before permission of leaving quarantine. To control this disease, all dairy farms (specially those of low milk production without obvious cause) should be investigated by performing ELISA to pooled bulk tank milk sample, if it gave positive result, all farm animals should be examined using PCR and ELISA followed by eradication of positive animals. In spite of high sensitivity of ABC technique, it is not recommended to be used now as its steps still complicated and expensive but with continuous work to simplify its steps and decrease its cost, may be it will the ideal technique of diagnosis of this disease in the future.

Key words: *Bovine Leukosis, Bovine Leukemia Virus (BLV), ELISA, Polymerase Chain Reaction (PCR), Avidine Biotin Peroxidase Complex (ABC).*

INTRODUCTION

In 1871, the observation of yellowish nodules in the enlarged spleen of a cow was considered to be the first reported case of bovine leukemia (enzootic bovine leukosis). The etiological agent of this worldwide lymphoproliferative disease is bovine leukemia virus (BLV), also termed

bovine leukosis virus, that primarily affects lymphoid tissues of animals. BLV belongs to the delta retrovirus genus which also includes the related human T-lymphotropic virus type 1 (HTLV-1) (Beier *et al.,* 1998; Ott *et al.,* 2003; Gillet *et al.,* 2007; Kohara and yokomizo, 2007). The disease causes great economic losses allover the world (Fulton *et al.,* 2006; Tiwari *et al.,* 2007) and usually infect dairy cattle. Usui *et al.,* (2003) in their study recorded 3.3% BLV-seropositive cattle in dairy farm while all examined beef cattle were seronegative for BLV. This disease infects cattle, sheep and water buffaloes (Meas *et al.,* 2000; Willems *et al.,* 2000). The disease transmitted through colostrums, milk (Meas *et al.,* 2002) and rectal palpation (Kohara *et al.,* 2006) while transmission through semen of infected bulls hadn't proven yet (Choi *et al.,* 2002; Meas *et al.,* 2002).

BLV induces a chronic infection in cattle, which develop in three possible pathological forms: asymptomatic course, persistent lymphocytosis (PL) and lymphosarcoma (Trono *et al.,* 2001; Debacq *et al.,* 2003). Infected cattle were classified into lymphocytotic and nonlymphocytotic groups. Nonlymphocytotic cattle comprised 2 subgroups: a group with high proviral load and strong immune response, and a group with low proviral load and weaker immune response. Nonlymphocytotic cattle with high or low proviral load could be efficient transmitters (as efficient as lymphocytotic cattle), but most cattle with low proviral load do not develop anti-BLV antibodies so, it couldn`t be detected serologically (Gutierrez *et al.,* 2001; Juliarena *et al.,* 2007). Viral spread by mitotic cell division accounts for most of the proviral loads during the asymptomatic phase has occurred (Florins *et al.,* 2007).

Once infected, cattle remain virus carriers for life and start to show a serological reaction within a few weeks after infection. Eradication and control of the disease is based on early diagnostic and segregation of the carriers (Suh *et al.,* 2005). The agar gel immunodiffusion (AGID) test has been the serological test of choice for routine diagnosis of serum samples for several years. BLV constitutes a serious sanitary problem for dairy producers in many countries. This problem increased by lack of rapid accurate diagnosis as most laboratories still depend on the official AGID which proved to has poor sensitivity as routine test for diagnosis of BLV Nevertheless, in more recent years, the enzyme-linked immunosorbent assay (ELISA) has replaced the AGID for large scale testing (Trono *et al.,* 2001).

The ELISA using either milk or serum to detect BLV-infected animals had comparable sensitivity and specificity with the official AGID and considered the most widely accepted test for the serological diagnosis of BLV infection. Therefore, ELISA can be used as an alternative test in

monitoring and control programs of BLV (Monti *et al.*, 2005a; VanLeeuwen *et al.*, 2005; VanLeeuwen *et al.*, 2006). and is advisable as a method of choice for screening investigation (Kurdi *et al.*, 1999).

The practical application of polymerase chain reaction (PCR) for the diagnosis of bovine leukemia virus (BLV) infections in naturally infected cattle was evaluated and compared to serological tests, the PCR was definitely found to be a more sensitive method, yielding the highest number of positive results (10% more compared t0 ELISA, and 17.7% more compared to AGID) (Fechner *et al.*, 1996). Many authors have recorded the sensitivity of PCR in comparison with other diagnostic tests of BLV (Nagy *et al.*, 2003; Kohara *et al.*, 2006)

BLV-infected cattle with low, transient or without BLV-antibody titers are difficult to identify as BLV-infected. These animals may be sources for new infections. Therefore, Monti *et al.*, (2005a) concluded that there is currently no reference test capable of serving as a gold standard technique for diagnosis of BLV. Although KSA imported large number of dairy cattle and has many large dairy farms allover the country, no studies have been conducted yet to determine the suitable and sensitive test for diagnosis of BLV infection. Also, there is no routine examination of imported dairy cattle for BLV before permission of introducing of these animals into KSA. So, it was the aim of the study to compare the suitability and sensitivity of enzyme-linked immunosorbent assay (ELISA) as serological test, polymerase chain reaction (PCR) as molecular biology-based assay and avidine biotin complex (ABC) immunoperoxidase as immunohistochemical technique to reach to a standard technique for diagnosis of BLV. Detection and elimination of infected cattle for control of this infection was another important aim of the study.

MATERIALS and METHODS

Animals:

300 dairy cattle imported from Australia were used in this study, out of them 200 were immediately imported and arrived to Jeddah airport. The remaining 100 cattle were imported and were added to dairy farm about one year before beginning of this work. All these cattle were apparently healthy.

serum:

5 ml blood was collected from each examined cattle to obtain serum for ELISA.

Blood:

5 ml blood was collected from each examined cattle to obtain viral nucleic acid (if present) for PCR.

Milk:

One milk sample taken from bulk milk tank in the dairy farm was obtained for performing ELISA.

Lymph node biopsy:

Lymph node biopsies from supra mammary lymph node were obtained using biopsy core needle (Stiefel Laboratories, Winterthur, Switzerland), Washburn *et al.*, (2007). These biopsies were used for detection of BL viral antigen by immunohistochemical staining using ABC method.

ELISA (in serum):

1- Serum samples were diluted twenty-five fold (1:25) with sample diluent (20µl serum + 480µl diluent).
2- Bovine Leukemia Virus (BLV) and Normal Host Cell (NHC) antigen coated plates and samples position were recorded (Table 1).

Table 1: Layout of the used ELISA plates.

	BLV	NHC	BLV	NHC	BLV	NHC	BLV	NHC	BLV	NHC	BLV	NHC
A	N	N	5	5	13	13	21	21	29	29	37	37
B	N	N	6	6	14	14	22	22	30	30	38	38
C	P	P	7	7	15	15	23	23	31	31	39	39
D	P	P	8	8	16	16	24	24	32	32	40	40
E	1	1	9	9	17	17	25	25	33	33	41	41
F	2	2	10	10	18	18	26	26	34	34	42	42
G	3	3	11	11	19	19	27	27	35	35	43	43
H	4	4	12	12	20	20	28	28	36	36	44	44

N=negative control P=positive control BLV=bovine leukemia virus NHC=normal host cell 1-44=samples

3- 200µl of negative control was dispensed into BLV wells A1 and B1 and into NHC wells A2 and B2.
4- 200µl of positive control was dispensed into BLV wells C1 and D1 and into NHC wells C2 and D2.
5- 200µl of diluted serum sample dispensed into the remaining wells.
6- Incubation for 90 minutes at room temperature (20ºC-25ºC).
7- Liquid contents of all wells were aspirated and plate washed 4 times with 300µl/well wash solution using automated washer, Well wash4 (Labsystems, A thermo Bioanalysis Company, Research technology Devision, Helsinki, FINLAND).
8- 100µl of Anti-Bovine IgG: HRPO (Horseradish Peroxidase) conjugate was added to each well.
9- Incubation for 30 minutes at room temperature (20ºC-25ºC).

10- Repeat step 7.
11- 100µl of TMB (Tetramethylbenzidine) substrate was dispensed into each well.
12- Incubation for 15 minutes at room mtemperature (20ºC-25ºC).
13- 100µl stop solution was dispensed into each well.
14- Spectrophotometer (Labsystems, A Thermo Bioanalysis Company, Research technology Devision, Helsinki, FINLAND) was blanked with air.
15- Optical density (OD) of samples and controls were measured at A650 wave length.
16- Calculation of results
    a- For the assay to be valid, the negative control mean (NCX) for the BLV wells and the NHC wells must be less than or equal to 0.200 and the difference {positive control mean (PCX) – NCX} for the BLV wells must be greater than or equal to 0.075.
    b- Calculation of NCX

$$NCX = \frac{A1 + B1}{2}$$

    c- Calculation of PCX

$$PCX = \frac{C1 + D1}{2}$$

    d- Calculation of sample to positive (S/P) ratio

$$S/P = \frac{\text{OD of sample - NCX}}{\text{PCX - NCX}}$$

    e- Calculation of sample to normal host cell (S/NHC) ratio

$$S/NHC = \frac{\text{OD of sample with BLV}}{\text{OD of sample with NHC}}$$

17- Interpretation of the results: Are shown in Tble 2.

Table 2: Interpretation of ELISA results.

	NEGATIVE	POSITIVE
S/P RATIO	Less than 0.500	Greater than or equal to o.500
S/NHC RATIO	Less than 1.80	Greater than or equal to 1.80

ELISA (in bulk milk sample):

Preparation of sample:

Whole milk sample were centrifuged for 15 minutes at 2000xg. Skim milk from underneath the fat layer was used as a milk sample.

Test protocol:

The same protocol as mentioned in serum except that negative,positive control and milk sample were added in a volume of 300µl, the first incubation of ELISA plate must be at 4ºC for 12-18 hours.
ELISA was performed according to manufacturer's (IDEXX, Idexx laboratories inc, Westbrook, Maine 04092 ,USA) directions.

PCR:

a-Nucleic acid extraction

Viral nucleic acid was isolated by a modified method of the procedure described by Chomczynski and Sacchi (1987). Briefly, 5µl purified leukocytes from examined animal was mixed with an equal volume of guanidine isothiocyanate lysis buffer (8M guanidine isothiocyanate, 50mM sodium citrate, 100 mM 2-mercaptoethanol, 1% sarkosyl). The solution was sequentially mixed with the following: 1µl sodium acetate (pH4), 10µl phenol (pH4.3), 2µl chloroform. The mixture was centrifuged at 16.000 g for 15 min, and the aquas phase was obtained and combined with equal volume of isopropanol to precipitate the nucleic acid. After centrifugation the resulting pellet was washed with 75% ethanol, dissolved in water and kept at –86ºC till use.

b-Primer design

Primers were selected according to genetic map of BLV (Oblap *et al.*, 1997; Reichert and Stec 1999; Monti *et al.*, 2005b; Felmer *et al.*, 2005; Hemmatzadeh 2007). Sequences and specifications of the used primers are shown in Table 3.

Table 3: Sequences and specifications of the used primers

PRIMER	SEQUENCE 5`-3`	POAITION	EXPECETD VIRUS
1	TCT AGC TCA GCT CAG TAG CCA	2948 – 2968	BLV (Brazilian subspecies)
2	CTA CCT TGC AGA TCT CAT C	2984 – 3002	BLV (Australian subspecies)
3	GCT TGT CGA AGC TCT GCA ATG C	3164-3185	BLV (Japanese subspecies)

c-PCR amplification of viral nucleic acid

Primers and viral nucleic acid were incubated at 75ºC for 10minutes and quickly cooled on ice for 10 min to allow annealing of the primer to nucleic acid. Then added to PCR reaction mixture which contained 1X PCR buffer (500mM Kcl; 100mM Tris-Hcl,pH8.3; 15mM Mg Cl2), 0.25mM dithiothreitol, 1mM of each deoxynucleotide (dATP, dCTP, dGTP, dTTP)

and 2.5 unit of amplitaq (Perkin-Elmer Cetus Corp., Norwalk,CT) in a reaction volume of 100µl. The PCR reaction was 30 cycles with the following reaction parameters: template denaturation at 94ºC for 1min, primer annealing at 55ºC for 1min and extension at 72ºC for Imin. A single final extension step was done at 72ºC for 10 min to complete the amplification reaction.

d-Analysis of PCR products

Amplified PCR products were analyzed by electrophoresis on 1.5% agarose gel (Sambrook *et al.*, 1989) and the specific nucleic acid band was identified {positive results were detected by presence and location of white band (Fig. 1)}.

PCR was performed according to method described by Reichert and Stec, (1999).

ABC:

Lymph node biopsies were examined for detection of BLV viral antigen using Avidin Biotin Complex (ABC) immunoperoxidase method as described by Hsu *et al.*, (1981) and Ikeda *et al.*, (2005) as follow:-

1-Paraffin sections from examined lymph node were cut at 6µm and picked up from warm water (40ºC) to Poly-L-Lysine coated slide and dry overnight in an oven at 37ºC.

2-Sections were deparaffinized and rehydrated by sequential immersion of the slides in xylene followed by graded concentrations of ethanol and then tap water.

3-The sections were treated for proteolysis with phosphate buffered saline (PBS, pH 7.6 prewarmed to 37ºC) containing 0.25% trypsin (Fluka, Buchs, Switzerland) and 0.02% $CaCl_2$ for 60 min at 37ºC.

4-After proteolytic treatment, the sections were washed three times each for 5 min in Tris-PBS (pH 7.6).

5-Endogenous peroxidase activity was blocked by immersion of slides in solution of 0.05%H_2O_2 in methanol for 30 min at room temperature (RT).

6-Before incubation with primary specific monoclonal antibodies (Tibueg, Netherlands) against BLV (gp 51), the sections were overlaid with normal rabbit serum diluted 1:10 in PBS for 20 min at RT.

7-The monoclonal mouse antibodies against BLV were applied diluted 1:100. Primary antibodies were incubated with the sections for 18 hours in humid chamber at 4ºC.

8-The slides were washed three times in Tris-PBS for 5 min each.

9-The biotinylated secondary rabbit anti-mouse-immunoglobulin (Amersham, Little Chalfont, Buckinghamshir, UK) diluted 1:200 in PBS were applied for 30 min at RT.

10-After washing in Tris-PBS three times for 5 min, the sections were incubated with ABC complex which prepared according to manufacturer's direction (Vectostain Elite ABC Vector Laboratories, Burlingam, CA, USA) for 30 min at RT.

11-Antigen binding was demonstrated with a solution of 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB, Fluka) in Tris-PBS containing 0.01% H_2O_2 (prepared immediately before use) as peroxidase substrate for 5 min at RT.

12-All sectrions were counterstained with Mayer's hematoxylene, dehydrated with a series of alcohol, cleared with xylene and a cover slide was applied with Entellan (Merck, Darmstadt, Germany).

13-The specificity of the immunohistochemical reactions was checked by replacing the primary antisera with non-immune sera or by omitting the secondary antibodies or the ABC solution. Positive results were detected by presence of brown staining of viral antigen (Fig.2).

RESULTS

(I) Imported cattle in quarantine

1-ELISA

16 cattle were positive and 184 were negative to BLV-antibodies.

2-PCR

21 cattle were positive and 179 were negative to BL viral nucleic acid.

The positive cattle were belong 3 subgroups, Australian (16 cattle), Japanese (4 cattle) and Brazilian (one cattle).

3-ABC

24 cattle were positive and 176 were negative to BLV antigen.

(II) Imported cattle in dairy farm

1-ELISA

a-ELISA (serum samples)

a- First: 6 cattle (5 of them from low milk producer cattle) were positive and 94 were negative to BLV-antibodies.
b- Second: No change from the result of the 1st examination
c- Third: 8 cattle (7 of them from low milk producer cattle) were positive and 92 were negative to BLV-antibodies.

b-ELISA (pooled bulk tank milk sample)

The pooled bulk tank milk sample which represent the whole farm was positive to 3 successive examinations.

2-PCR

a- First: 7 cattle (6 of them were from low milk producer cattle) were positive and 93 were negative to BL viral nucleic acid.
b- Second: 8 cattle (7 of them were from low milk producer cattle) were positive and 92 were negative to BL viral nucleic acid.
c- Third: No change from the result of 2nd examination
d- Positive animals were belong 3 subgroups, Australian (4 cattle), Japanese (2 cattle) and Brazilian (one cattle).

3-ABC

a- First: 9 cattle (8 of them were from low milk producer cattle) were positive and 91 cattle were negative to BL viral antigen.
b- Second: 11 cattle (10 of them were from low milk producer cattle) were positive and 89 cattle were negative to BL viral antigen.
c- Third: No change from the result of 2nd examination.

Results of laboratory investigation are summarized in Table (4), results of PCR are shown in fig.1and results of ABC are shown in Fig.2

Table 4: Results of laboratory investigation.

	ELISA						PCR						ABC					
	1st		2nd		3rd		1st		2nd		3rd		1st		2nd		3rd	
	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Cattle in quarantine	16	184	No		No		21	179	No		No		24	176	No		No	
Cattle in a farm																		
a-low milk producer cattle	5	45	5	45	7	43	6	44	8	42	7	43	8	42	10	40	10	40
b-Randomly selscted cattle	1	49	1	49	1	49	1	49	1	49	1	49	1	49	1	49	1	49

No=not performed

Fig. 1: Results of PCR
Lane1 (DNA size marker).Lane2 (BLV,Australian subspecies).Lane3 (BLV, Japanese subspecies).Lane4 (BLV, Brazilian subspecies).Lanes 5-8 (negative results).

Fig. 2: Immunohistochemical assay using ABC technique in supra mammary lymph node. Brown staining represent BL viral antigen.

DISCUSSION

BLV is one of the most important infectious disease agents that can be harbored in apparently healthy animals, (Tiwari *et al.*, 2007). In the present study all animals proved to be infected with BLV were apparently healthy. This observation clearly showed the importance of laboratory investigation of dairy cattle for diagnosis of bovine leukosis. Therefore, ELISA, PCR and ABC techniques were used for diagnosis of BLV in the present work.

During this work two cattle change from negative to positive cases which indicated the transmission of infection from infected to healthy animals even in absence of any clinical signs. BLV transmitted during asymptomatic phase (Florins *et al.*, 2007). This change was recorded by ABC method and PCR assay before detection by ELISA.BLV proviral DNA were detected by PCR 1 to 5 weeks earlier than detection of the antibodies by serological tests (Beier *et al.*, 1998; Kohara *et al.*, 2006; Nagy *et al.*, 2007). These authors considered PCR not only a useful screening test

for early diagnosis of natural BLV infection but also the most rapid method for the early detection of BLV infection in cattle.

Results of this study proved that PCR more sensitive than ELISA where PCR detected BLV viral nucleic Acid in 30 cattle while ELISA detected Antibodies against BLV in 24 cattle only. PCR is a highly sensitive method and might be successfully used and economically advantageous for different practical applications in detection of BLV infection in naturally infected cattle. PCR-technique compared to the serological tests proved to be much more sensitive (Fechner *et al.,* 1996; Beier *et al.,* 1998; Kurdi *et al.,* 1999; Konnai *et al.,* 2003; Nagy *et al.,* 2003; Kohara *et al.,* 2006). This difference in sensitivity between ELISA and PCR may be explained by the conclusion of Gutierrez *et al.,* (2001) and the results of Juliarena *et al.,* (2007) who proved that most cattle with low proviral load do not develop anti-BLV antibodies and observed that all cattle that had positive results for the PCR had BLV antibodies, but cattle with consistantly low antibody titers required examination of sequential DNA samples to detect viral sequences

However, 2 cattle were ELISA positive and PCR negative, 8 cattle were PCR positive and ELISA negative. At the same time, 11 negative cattle with ELISA and 5 negative cattle with PCR were positive with ABC method. These results proved that ABC is more sensitive than PCR and ELISA and also showed that PCR or ELISA alone is not sufficient for accurate diagnosis of BVL. Klintevall *et al.,* (1994) in their experimental study observed some infected animals without detectable levels of BLV proviral DNA in blood and without circulating antibodies. Of 18 cattle imported from the Slovak Republic and kept in a quarantine stable, four were found to be BLV provirus positive by PCR, while serological tests indicated one animal positive and three negative. Therefore, it is impossible to prevent the spread of the infection from one country to another by serological testing only (Fechner *et al.,* 1996; Trono *et al.,* 2001). The findings of persistently seronegative PCR positive and seropositive PCR negative cattle indicate that further work is needed to overcome the problem of diagnosis of BLV. Present serological screening methods may not have sufficient sensitivity for determining BLV status in some circumstances. PCR assay alone is unreliable for routine detection of BLV in herds with high prevalence of the disease (Jacobs *et al.,* 1992; Nagy *et al.,* 2003). Schell *et al.,* (2004) observed leucotic and tumorous alterations which accompanied to BLV infection while blood serum was negative for antibodies directed against BLV. Beier *et al.,* (1998) and Nagy *et al.,* (2007) concluded that PCR is more sensitive than ELISA, therefore it is considered a useful tool to exclude or confirm BLV-infection in cattle with doubtful

serological results and may be used to complement the serological tests in the diagnosis of BLV-infection.

PCR used in this work could detect and differentiate between three subspecies of BLV (Fig. 1) while ELISA couldn't differentiate between them, Australian subspecies was the most common among infected cattle. Different BLV provirus variants had been recorded in the examined animals. Kurdi *et al.,* (1999) recorded 3 subgroups of BLV. Belgian, Japanese and Australian subspecie of BLV were reported by Limanskii *et al.,* (2004) who observed Australian subspecies in 92% of infected cattle. Camargos *et al.,* (2007) concluded that BLV belonged to at least two phylogenetic clusters. Four different genetic groups of BLV were identified by phylogenetic analysis (Camargos *et al.,* 2002; Felmer *et al.,* 2005). These subgroups were identified by PCR (Monti *et al.,* 2005b; Hemmatzadeh, 2007). A serologic subgroup of bovine leukemia virus (BLV) has not been identified, whereas genetic diversity among BLVs has been reported by PCR (Beier *et al.,* 2001; Licursi *et al.,* 2003). Presence of the same subspecies of BLV in both animals in quarantine and farm animals (which imported from Australia and examined in this study) proved that these imported cattle harbor BLV before their arrival to KSA. Beier *et al.,* (1998) after their study concluded that cattle in the same area usually infected with the same BLV provirus variant.

In dairy farm examined in this study, most BLV-positive cattle were low milk producer. Ott *et al.,* (2003) recorded decrease in milk produced by dairy cattle infected with BLV.

In the present investigation, detection of antibodies against BLV in bulk tank milk sample by ELISA proved to be a good method for primary diagnosis of BLV in dairy farms. Examination of pooled milk samples with the ELISA provides a reliable, practical, and economic procedure for identification of BLV-infected herds (Gutierrez *et al.,* 2001).

From results recorded in this study, I can conclude that ABC is more sensitive than PCR and ELISA and PCR more sensitive than ELISA .However, accurate diagnosis of BLV needs achievement of two of the three techniques used in this work. From practical point of view, imported dairy cattle should be routinely examined by PCR and ELISA before permission of leaving quarantine. To control this disease, all dairy farms (specially those of low milk production without obvious cause) should be investigated periodically by performing ELISA to pooled bulk tank milk sample, if it gave positive result, all farm animals should be examined using PCR and ELISA followed by eradication of positive animals. In spite of high sensitivity of ABC technique, it is not recommended to be used now as its

steps still complicated and expensive but with continuous work to simplify its steps and decrease its cost, may be it will the ideal technique for diagnosis and epidemiological studies of this disease in the future.

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