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CLINICAL, LABORATORY AND MOLECULAR EPIDEMIOLOGICAL STUDIES ON A LIMITED OUTBREAK OF ENZOOTIC BOVINE LEUCOSIS IN UPPER EGYPT

BY

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SUMMARY

In 1989,220 Holstein Friesian cattle (212 heifers and 8 bulls) were imported from Minnesota, USA to form a closed dairy herd in Arab El-Aoumar, Assiut, Upper Egypt. In November 1996 the population had built up to 440 animals. At that time some abnormal signs such as loss of weight, decreased milk yield, external lymphadenopathy and decreased appetite were observed in this farm.

Serological screening by Enzyme linked immunosorbent assey (ELISA) revealed a seroprevalence of antibodies directed against Bovine Leucosis Virus (BLV) of 37.7% in cattle under 2 years old and of 72.8% in animals more than two years old. Diagnosis was confirmed by the detection of BLV proviral DNA by polymerase chain reaction (PCR) using primers, amplifying a fragment of the env gene. Out of 22 tested leucocyte fraction from individual animals, 15 were positive showing a BLV-specific amplicon of 440 bp. The analysis of the amplicon for restriction fragment length polymorphisms and DNA sequencing results allowed to type the isolates. Since this is the first recorded case of enzootic bovie leucosis in upper Egypt, strict quaratine measures were adopted and all serologically positive animals of the herd culled.

INTRODUCTION

Enzootic bovine leukosis is a systemic, malignant neoplasia of the reticuloendothelial system of cattle caused by Bovine Leukaemia Virus (BLV), a

retrovirus which is closely related to the human T cell leukaemia viruses HTLVI and HTLV II (Burny et al., 1988, Coffin et al., 1991; Radostits et al.,1995). While BLV infection typically leads to a strong humoral immune respose (Mammerickx et al., 1987), only a small precentage of cattle infected with BLV develop a persistig lymphocytosis or tumors (Ghysdael et al., 1984; Johnson and kaneene, 1992). Neoplasias induced by BLV are monoclonal and are characterised by the integration of provirus into the genome of the host cell. However, the expression of viral genes is not required for the maintenance of the transformed state (van den Broeke et al. 1988).

Epidemiological observations indicates that enzootic bovine leucosis is predominantly horizontally transmitted (Van der Maaten and Miller, 1990). Studies performed in cattle herds with a high proportion of BLV-infected cattle and with high tumor incidence showed that 14% of the newborn calves had congenital infections (Ferrer et al.,1976) . Blood transfusion and artifical insemination as well as blood sucking vectors are incriminated in the transmission of BLV (Monke, 1986; Perino et al.,1990). The risk of BLV transmission during reproductive tract examination of cattle is related to frequency of rectal palpation and the age of the animals (Hopkins et al., 1991).

Cattle with a high antibody titre appear to be more important as a source of infection than animals with low antibody titres (Koyama et al., 1988). Removal of all seropositive cattle from a herd quickly reduces the prevalene of BLV infection (Johnson and Kaneene, 1992).

In this publication, we describe the first known case of enzootic bovine leukosis in Upper Egypt which occurred in Arab El-Aoumar, Assiut Governorate.

MATERIALS AND METHODS

History

In 1989,220 Holstein Friesian cattle (212 heifers and 8 bulls) were imported from Minnesota, USA to form a closed dairy herd in Arab El-Aoumar, Assiut, Upper Egypt. Until November 1996, when the first clinical manifestations of enzootic bovine leukosis were observed. At that time the population had built up to 440 animals.

Blood and serum samples

The whole herd (n=440) was bled. Whole blood without anticoagulant was used to obtain serum samples employed in antinbody detetion From selected aninmals (n=22) 10 ml whole blood samples containing Ethyline diamine tetra acetic acid (EDTA) as an anticoagulant were taken and buffy coats were prepared for PCR and DNA sequenicing.

Serological test

A commercially avialable Enzyme-linked immuosorbent assay (ELISA) was used according to the manufacturer's instructions to detect seropositive animals (CHEKiT -leucotest; Dr. Bommeli AG, Bern, Switzerland).

Preparation of leukocytic fraction,

To seprate the leukocytec fraction, 10 ml blood samples (with EDTA) were centrifuged at 2000 r. p. m. for 15 minutes. The supernatant was discarded and

about 5 ml 0.04% (w/v) NH4Cl dissolved in destilled water were added to the sediment and shake gently to lyse erythrocytes. The suspension was centrifuged at 2000 r. p. m. for 10 minutes and the white pellet kept at -20 ^{o} C until further use.

Isolation of DNA

DNA was isolated from the leukocyte fractions using commercial kits according to the manufacturer's instructions (Nuclesospin Blood; Macherey-Nagel GmbH & Co KG, Duren, Germany).

BLV-provirus nested-PCR

Oligonucleotide primers for PCR were designed according to sequence published data (Sagata et al., 1985; Naif et al., 1990, 1992). The primers used in this study were obtained from MWG Biotech (Ebersberg, Germany) and had the following sequences:

env $^{-1}$ (5032)' 5'-TCT GTG CCA AGT CTC CCA GAT A-3'

env $^{-2}$ (5608)' 5'-AAC AAC AAC CTC TGG GAA GGG T-3'

env $^{-3}$ (5099)' 5'-CCC ACA AGG GCG GCG CCG GTT T-3'

env $^{-4}$ (5521)' 5'- GCG AGG CCG GGT CCA GAG CTG G-3'

Reaction mixtures containing 5 'ul of 10 x PCR buffer (500 mM KCi; 100 mM Tris-HCi; pH 9.0; 1 % Triton X-100; Promega, Mannheim, Germany), 0.15 ul of 75 uM dNTP solution, 3 ul 25 mM MgCl2 (Promega). 1.25 ul of a 25 pM ul^{-1} solution of each primer, 1.25 units Tag polymerase (promega, Madison, USA) and 25 ul leuocyte DNA sample were prepared and filled up with distilled water to a total volume of 50 ul.

The use of primer pair env 5032/env 5608 (outer primers) results in the amplification of a 598 bp fragment. A second (nested) PCR was carried out with the inner primer pair env5099/env 5521 resulting in the amplification of a 444 bp fragment. All amplification reactions were performed in a DNA thermal cycler 480 (Perkin - Elmer Cetus, Inc., Weiterstadt, Germany). First, an initial incubation at 94 ^{o}C for 2 minutes was performed followed by 40 cycles consisting of denaturation at 95 ^{o}C for 30 seconds; primer annealing at 62 ^{o}C (primer env 5032/env 5608r) or 70 ^{o}C (primer env 5099/env 5521r) for 30 seconds and primer extension at 72 ^{o}C for 60 seconds.

For the first and last five cycles of the PCR the extension time was increased to 75 seconds followed by final extension at 72 ^{o}C for 4 minutes. For the second round of PCRs (nested), 3 ul were taken from the first amplification and used as template in the second PCR. To visualize PCR products, 20 ul of amplified mixture were electrophoretically separated in 2% agarose gels followed by ethidium bromide staining.

DNA sequencing

Sequences of purified PCR products were determined by use of the fluorescent dye deoxy-terminator cycle sequencing kit (Perkin-Elmer) and the internal env PCR primers. The reaction was performed within 25 cycles of incubation at 96 ^{o}C/30 sec, 50^{o}C/4 sec in a PTC-100 thermocycler with a heated lid (MJ Research, ic,). Sequencing products were purified using the QlAquick nucleotide removal kit (QiAGEN), vacuum-dried and stored at- 20 ^{o}C until

analysis in an ABi Prism 377 DNA sequencer (Applied Biosystems). The nucleatide sequence of the env gene of EBL virus was early analysed by Rice et al. (1984).

Restriction analysis

Three restriction enzymes (Bam HI, Bcll and PvuII; Gibco BRL, Eggenstein, Germany) were used for further identification of the virus strain. Equal amounts of amplificate were incubated with the restriction endonuclease under the appropriate conditions recommended by the manufacturer, the resulting fragments electrophoretically separated in 2% agarose gels and stained with ethidium bromide.

Statistical analysis

EPi-iFO 6.03 (Centers for Disease Control and Prevention, Atlanta, GA, USA, and World Health Orgaisation, Geneva, Switzerland) was used for statistical analysis.

RESULTS

Serology

When the herd was tested for antibodies to BLV, 230 animals reacted positive in the ELISA which corresponds to a total seroprevalence of 52.3 % . In the group of animals younger than 2 years (n=257), 97 carried antibodies to BLV, corresponding to a seorprevalene of 37.7% in this age stratum (Table 1). 133 cattle older than 2 years were BLV-positive by ELISA serology (seroprevalence 72.7%). The difference in the seorprevalence in both age groups is highly significant (Odds ratio 4.39 [95% confidence interval 2.85-6.77]; relative risk 1.93 [95% confidence interval 1.61-2.31]; $p< 0.0001$).

Clinical symptoms

The clinical symptoms observed during this outbreak were loss of appetite, decreased milk yield, decreased production and enlargement of superficial lymph nodes specially precrural lymph nodes. Paleness of the mucous membranes, general weakness and loss of body weight were also observed. Both male and female infertilities were noticed. Some cattle suffered from exophthalmia, other showed signs of posterior paresis. Internal lymphadenopathy was commonly observed during rectal palpation and post - mortum examination .The frequency of the different clinical manifestations in 230 serologically positive cattle is illustrated in Table 2.

Detection of EBL Provirus by polymerase chain reaction (PCR)

To confirm the serological diagnosis by direct proof of proviral BLV DNA, leukocyte preparations from 22 animals were tested by PCR using primers specific for the env gene. in samples from 15 animals BLV -ev specific amplification products were detected (Table 3, Fig. 1 and 2).

Molecular typing

The analysis of 15 env amplicons for restriction fragment lenght polymorphisms (RELP) using the restriction endonucleases BamHI, Bcl1 and PvuII revealed that 12 samples yielded the same pattern as isolated first charaterized by Coulston and co-workers (1990) in Australia. One isolate produced the same patterns as the Belgian variant (Rice et al., 1984) (Fig. 1). The pattern obtained with two isolates was different from those of the reference strains used. This result was confirmed by DNA sequencing of two typial amplicons from the herd. DNA sequencing

Table 1 : Results of serological screening of 440 Holsten Friesian cattle by Enzyme linked immunmdsorbent assay (ELISA) .

Age groups	Total	ELISA		
		pos.	neg.	Prevalence(%)
< 2 Years	257	97	160	37,7
> 2 Years	183	133	50	72,7
Total	440	230	210	52,3

Table 2 : Frequency of predominant clinical manifestions of EBL in 230 serologically positive cattle .

Clinical manifestation	Frequency	Frequency %
Loss of weight	189	82,2
Decreased milk yield	184	80,0
Internal lymphadenopathy	169	73,5
Infertility	81	35,2
Diarrhoea	76	33,0
Fever	58	25,2
Posterior paresis	39	16,9
Respiratory involvement	36	15,7
Cardiovascular involvement	32	13,9
Exopthalmia	16	6,9
Bloat	10	4,3
Constipation	6	2,6

Table 3: Serological and molecular results of seleted samples collected from Holstein Friesian cattle .

samples	Serology	BLV-Provirus-test	RELP-(env)			Sequence
	ELISA	PCR	isolate-identity			env.-Amp
	Pourquier	**env**	**Auster**	**Belg.**	**unknown***	
1	Pos.	Pos.	Pos.			Pos.
1	Pos.	Pos			Pos.	Pos.
5	Pos.	Pos	Pos.			N.D
1	Pos.	Pos	Pos.			N.D
1	Pos.	Neg.				N.D
3	Pos.	Neg.	Pos.			N.D
3	Neg.	Neg.				N.D
5	Neg.	Pos.	Pos.			N.D
1	Pos.	Pos.		Pos.		N.D
1	Neg.	Pos.			Pos.	N.D
22	13/22.	15/22	12	1	2	2

Pos.: positive
Neg.: negative
N.D.: not done
RFLP: restriction fragment lenght polymorphismus
Austr.: Pattern as determined by COULSTON et al. (1990)
Belg.: Pattern as determined by RICE et al. (1984)
AGID. agar gel immune diffusion
: Polymerase chain reaction
PCR: see also sequencing results

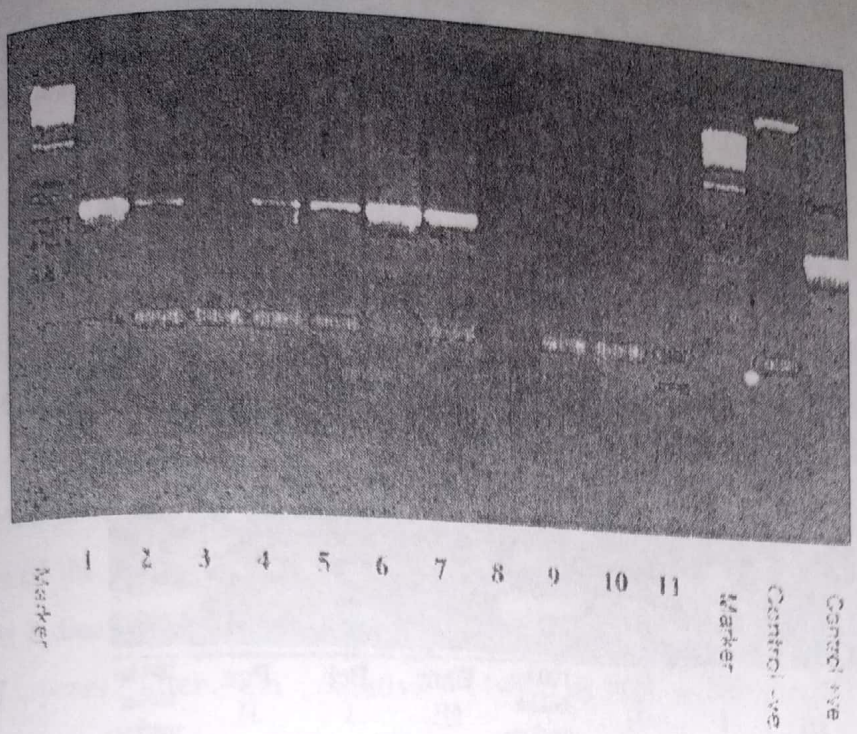

Fig. (1): PLV - provirus nested PCR using env-primer of leukocytic fractions (1-11) collected from Holstein Friesian cattle.

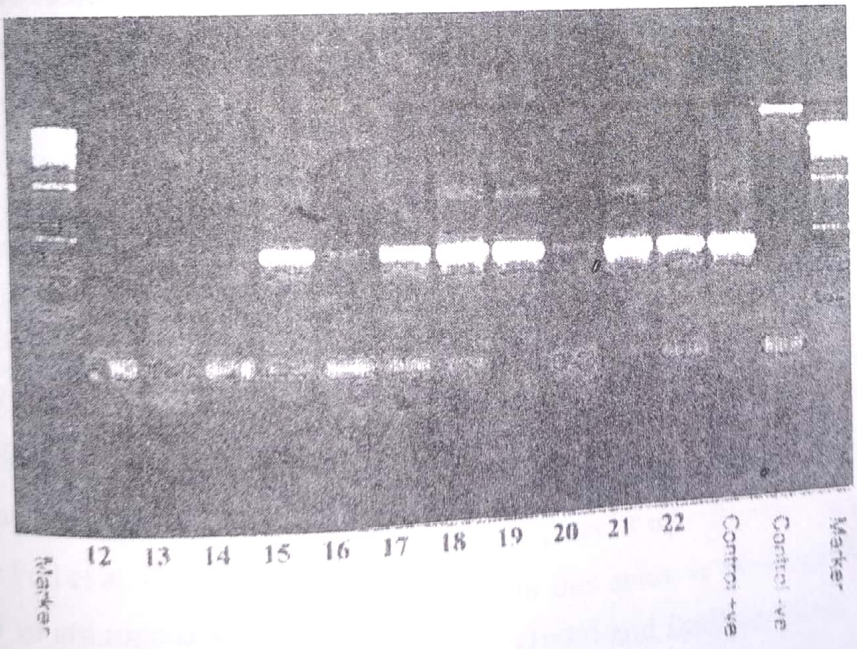

Fig. (2): PLV - provirus nested PCR using env-primer of leukocytic fractions (12-22) collected from Holstein Friesian cattle.

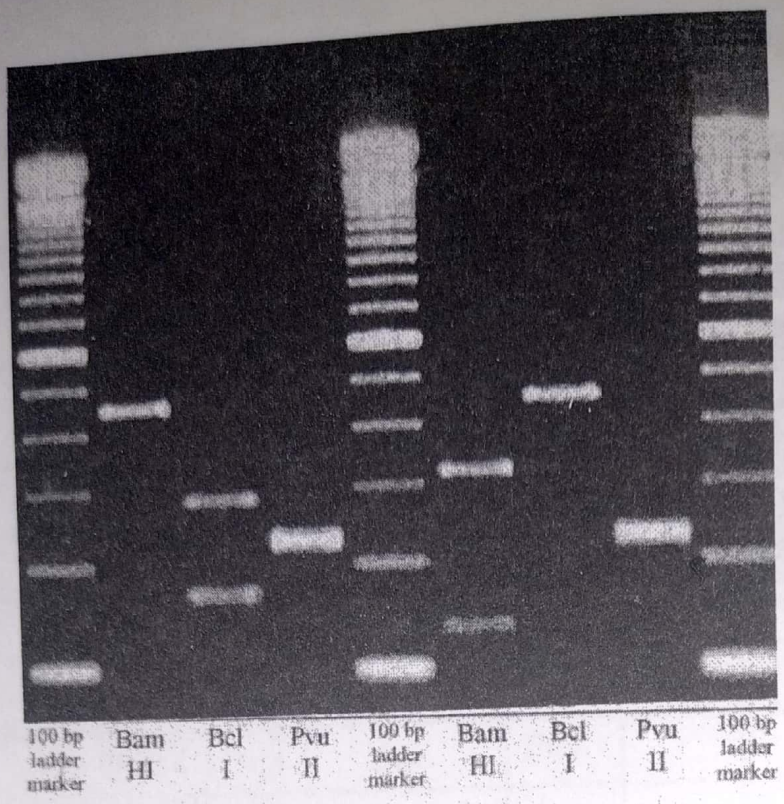

Fig. (3): Restriction analysis using Bam HI, BCLI and PVU II enzymes for identification of Belgian and Australian isolates of BLV.

confirmed the identity of the sequence of one of the isolates with that published by Coulston et al. (1990). One isolate with an unknown restriction pattern had a single point mutation at position 552 (T->C) which lead to the destruction of a PvuII site.

DISCUSSION

This report describes the first known outbreak of enzootic bovine leukosis in Upper Egypt. In a herd of Holstein Friesian imported in 1989 from Minnesota, USA, first clinical cases of the disease were recorded in November 1996. This indicated that the incubation period was about 7 years after the putative introduction of BLV-infected carrier cattle. The incubation period of enzootic bovine leukosis ranges between 4 and 5 years after introduction of first cases into a herd (Hungerford, 1990 and Roberts et al., 1985).

The high density of animals on the farm suggests that all kown modes of transmission including intrauterine infections were possible (Fechner, 1995). Multiple use of injection needles during vaccinations or treatments may have contributed to the transmission in the herd .

These routes are of great importance in the transmission of BLV (Kaaden & Lange, 1984; Van der Maaten & Miller, 1990). Furthermore, blood sucking arthropods may have served as vectors for the virus as suggested by Foil et al. (1989) and Perino et al (1990). The presence of arthropods suitable as vectors in the geographical area of the farm is well known, Favourable climatic conditions, especially high temperatures, and the presence of the large cattle herd may have helped some relevant insect to built up high population densitites.

The clinical characteristics of the outbreak is quite similar to the finding of Reed (1981) concerning the frequency of predominant clinical signs such as loss of weight, decreased milk prodution and external lymph adenopathy (Table 3).

The total prevalences found in the herd agree with those of Jimenez et al. (1995) who recorded a total prevalence percentage of 51% when testing 1879 herds in Costa Rica and found high prevalences in Holstein and Jersey breeds (65.7%). The BLV seroprevalence in cattle older than 2 years was almost twice as high as in younger animals. The association of age and increased seroprevalence was highly siginficant. The seroprevalence in a herd can be influenced (i) by the time individual aimals are 'at risk' for contracting an infection, (ii) by the time, they require to mount a humoral immune response to the virus, and (iii) by the persistence of antibodies., Obviously, older animals spent a longer time at risk in the study herd and were therefore more likely to become BLV infected.

Depending on the dose of infection it takes between 4 weeks and several years untill individual animals develop a detectable immune response (Roberts et al., 1985). Persisting maternal antibodies may lead to a certain overestimation of the prevalence in calves. The higher prevalence rate of infection in cattle more than 2 years of age than that under 2 years of age recorded in this study is in accord with Johnson and Kaneene (1992) and Detilleux et al. (1991) who concluded that the prevalence of EBLV infections is positively associated with age and that the prevalence of infection in cattle under 17-24 months of age is much

lower than inadult cattle. They further stated that the prevalence of infection increases sharply after 24 months of age when heifers join the dairy herd and are in close contact with older cattle.

Polymerase chain reaction is a recent and a good diagnostic tool for the detection and/or confirmation of BLV infection (Klintevall et al., 1993; 1994; Eaves et al., 1994; Fechner et al., 1997). The use of primer pairs for PCR with target sequences in the env gene allows to detect as few as 10 BLV provirus copies (Fechner et al., 1996). Primers to the gene env were selected because this region is highly conserved among different BLV provirus isolates (Mamou et al., 1990) but also because amplicon obtained from that region can be used for typing. In our study, 15 out of arbitrarily selected leukocyte preparations (n=22) were positive by PCR using the env primers.

Typing of the amplications by RFLP analysis and DNA sequencing of two representative samples revealed that the Egyptian isolates were identical or similar to an Australian isolate (Coulston et al. 1990). By contrast, in Europe a provirus variant seems to prevail which was found in Belgium and Frence (Rice et al., 1984; Mamoun et al., 1990) and Germany (Fechner et al., 1997).

In the U.S.A., approximately 10% of dairy cattle are estimated to be infected with BLV (Dube et al., 1997). Together with the fact imported animals were kept as a closed herd in Egypt with no influx from other Egyptian regions or from abroad, our findings are in accord with assumption that clinically healthy BLV- infected cattle were already imported when the herd was formed. Also the case history of the outbreak with the time span that elapsed before the onset of clinical disease in the herd supports this view. Similar outbreak of enzootic bovine leukosis associated with the importation of cattle were reported from Ireland (Kavanagh 1978), The Netherlands (De Fries, 1978) and Saudi Arabia (Hafez et. 1990) .

In conclusion it is recommended that counteries free from BLV infections adopt safety procedures which aim at the prevention of the import of infected cattle from abroad. Such measures should include an appropriate quarantine period upon arrival and serological testing for BLV antibodies while the animals are kept in quarantine. Recently , it was suggested to include PCR in the routine laboratory testing of breeding cattle that are assigned for trade (Blankenstein et al.,1998) .

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