

PARTIAL BLV RNAs ARE PRESENT IN PLASMA EXOSOMES OF BLV INFECTED ANIMALS

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ABSTRACT

Exosomes are membranous nano-vesicles of endocytic origin that are released by almost all cell types and play an important role in cell-to-cell communication. Many researches have been carried out to illustrate the role of exosomes in the life cycle and pathogenesis of RNA viruses, however, information regarding their role in Bovine leukemia virus (BLV) is meagre. Herein we tried to investigate the role of exosomes isolated from plasma in BLV life cycle. Exosomal fractions were isolated from 18 fresh BLV-positive plasma samples by size exclusion chromatography (SEC) and then were tested using western blotting, RT-PCR and sequence analysis. Our results showed that absence of BLV enveloped protein (env) in all tested exosomal fractions, on the other hand around 22% of samples showed the presence of partial BLV RNA in their exosomes. From this approach, it was emphasized that the exosomes may incriminate in BLV pathogenesis through transfer of viral RNA from infected to uninfected B cells.

Keywords: Exosomes, BLV, RT-PCR, sequence analysis, Western-blot.

Exosomes play an important role in cell-to-cell communication and influence both physiological and pathological processes together with the whole process of pathological tumor metastasis (An *et al*, 2015; Lin *et al*, 2015). Exosomes are small (50-90 nm) cup shaped membrane nano-vesicles of endocytic origin that are released into the extracellular environment through the fusion of multi-vesicular bodies (MVB) with the plasma membrane (Van Niel *et al*, 2006; Conde-Vancells *et al*, 2008; Zomer *et al*, 2010). They are released by almost all cell types and have been confirmed in all biological fluids such as bronchoalveolar fluid, cerebrospinal fluid, blood, urine and saliva etc. (Zech *et al*, 2012; Sharma *et al*, 2016).

Bovine leukemia virus (BLV) is a member of RNA viruses which belongs to family *Retroviridae*, genus *Deltaretrovirus*. BLV is the causative agent of enzootic bovine leucosis (EBL) that has a worldwide distribution and can result in economic losses to the cattle industry (Juliarena *et al*, 2013; Nekouei *et al*, 2015). BLV infection is transmitted mainly by horizontal routes but also can be transmitted vertically through ingestion of colostrum or in utero infection (Gillet *et al*, 2013; Bartlett *et al*, 2014; Mekata *et al*, 2015; Sevik *et al*, 2015). Most BLV-infected cattle (about 70%) do not show any clinical symptoms, but only about 30% of them develop persistent lymphocytosis (PL) and 1-5%

of the infected cattle develop malignant B-cell lymphosarcomas causing (EBL) (Kettmann *et al*, 1980; Balic *et al*, 2012; Kim *et al*, 2015; Ohno *et al*, 2015).

Several studies have been carried out to investigate the role of exosomes in pathogenesis and life cycle of RNA viruses and it has been demonstrated that exosomes released from RNA viruses' infected cells as Human Immunodeficiency Virus (HIV), Hepatitis C Virus (HCV), human T-cell lymphotropic virus (HTLV), Dengue Virus (DENV) and BLV harbor and deliver many regulatory factors including viral RNA, miRNA and proteins to neighboring cells, helping to establish productive infections and modulating cellular responses (Yamada *et al*, 2013; Chahar *et al*, 2015).

Since the BLV pathway within the body of infected animal is still mysterious besides no reports about the role of exosomes in BLV pathogenesis, we tried to examine the role of exosomes in BLV infection through detection of BLV proteins and RNA in exosomes isolated from plasma of BLV infected animals.

Material and Methods*Animals and samples*

Holstein cows (1.6-8.5 years old) were screened for BLV infection using ELISA targeting the BLV gp51 enveloped protein according to manufacturer's instruction with some modification (JNC Co, Ltd, Tokyo, Japan) (<http://www.jnccorp.co.jp/product/chemical/>

Table 1. Primers' used in the present study and their details.

Name	Sequence	length	T _m (°C)	GC (%)
RT-PCR				
Env 5032 F	TCTGTGCCAAGTCTCCCAGATA	22	57.5	50
Env 5608 R	AACAACAACCTCTGGGAAGGGT	22	58.7	50
env 5669 F	CACCCCTATCTCCCTGGTTAATCTC	25	58.1	52
env 5788 R	ATTAATTCCAGTGAGCCCCACTG	23	57.1	47.8
Full env F	ATGCCTAAAGAACGACGGTCCCG	23	61.2	56.5
Full env R	AGGGCAGGGTCGGAGGTTGATGT	23	64.6	60.9
AS1-S/L F*	TCCCTTCTGTTTCCACAACG	20	55.1	50
AS1-L F*	CCCCTAAACCCGATTCTGAT	20	54.1	50
AS2 F*	CCCAGTCTCTTCTGGTGTCC	20	57.1	60
LTR R*	CAGAGGACCGGAGATAGAGC	20	56.4	60
Anti-Orf F*	CACCATGGATCCCGAAGAGCTTGAA	25	60.8	52
Anti-Orf R*	ACGGAGGTTTCAGAATTTCTCTCT	22	55.8	45.5
PCR and sequence analysis				
Env 5032 F	TCTGTGCCAAGTCTCCCAGATA	22	57.5	50
Env 5608 R	AACAACAACCTCTGGGAAGGGT	22	58.7	50

*Antisense primers

Table 2. Samples information with the result of ELISA, Real-time PCR, and DNA concentration for the plasma samples used for exosome isolation.

No	Species	Sex	Age/ month	ELISA	Proviral load Copies/50ng	DNA Conc.
1	Holstein	♀	56	+ Ve	3463	50ng/uL
2	Holstein	♀	59	+ Ve	4516	50ng/uL
3	Holstein	♀	102	+ Ve	2271	50ng/uL
4	Holstein	♀	36	+ Ve	3478	50ng/uL
5	Holstein	♀	28	+ Ve	4383	50ng/uL
6	Holstein	♀	34	+ Ve	4010	50ng/uL
7	Holstein	♀	70	+ Ve	5873	50ng/uL
8	Holstein	♀	94	+ Ve	2925	50ng/uL
9	Holstein	♀	60	+ Ve	2106	50ng/uL
10	Holstein	♀	56	+ Ve	4945	50ng/uL
11	Holstein	♀	56	+ Ve	3463	50ng/uL
12	Holstein	♀	99	+ Ve	3203	50ng/uL
13	Holstein	♀	52	+ Ve	3605	50ng/uL
14	Holstein	♀	81	+ Ve	4073	50ng/uL
15	Holstein	♀	20	+ Ve	4208	50ng/uL
16	Holstein	♀	35	+ Ve	2518	50ng/uL
17	Holstein	♀	36	+ Ve	>2 low	50ng/uL
18	Holstein	♀	40	+ Ve	>2 low	50ng/uL

animal/blv.html).

Total 18 BLV whole blood samples were selected and were divided into two parts; one part was centrifuged at 3000 RPM for 5 minutes at 4 °C to obtain plasma for exosome isolation and second part kept as whole blood for proviral DNA extraction for Real-time PCR (Table 2).

Extraction of BLV-proviral DNA from whole blood

The proviral DNA was extracted using a Wizard Genomic DNA purification kit (Promega, Fitchburg, USA) according to the manufacturer's instructions, then the DNA concentration was measured using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA).

Real Time PCR

Real-time PCR was carried out to quantify the BLV proviral load. The DNA concentration was diluted to 50 ng / μ l using Milli Q water (PCR grade water) before carrying out real- time PCR.

Cycleave® PCR Reaction Mix kit (Takara BIO INC. Cat # Y505A, Lot # AK1701) and Probe / Primer / Positive kit (Takara Biotechnology Co., LTD. Cat # CY 415) were used according to manufacturer instructions. The amplifications were performed in 10 μ l reaction volumes containing 5.0 μ l 2x cycleave PCR reaction, 0.2 μ l Probe / primer mix for BLV, 0.2 μ l Rox Reference Dye I, 0.5 μ l DNA template and 4.1 μ l Milli Q water.

Exosomes isolation

Exosomes were isolated from the fresh BLV-infected plasma samples for detection of BLV proteins and RNA using Extracellular Vesicle Isolation by Size Exclusion Chromatography on Drip Column kit (EV-Second kit 10/pK, GL Sciences, Tokyo, Japan, lot # E16G0024) according to manufacturer instruction. Plasma samples were filtrated using 0.22 mm filter before exosomes isolation to obtain pure exosomes population furthermore, each sample gave 12 exosomal fractions thus, 216 (12 fraction X 18 samples) fractions were examined.

Coomassie Brilliant Blue and Western Blot technique

Both techniques were carried out for detection of BLV proteins inside the isolated exosomes briefly as follow:

10 μ l exosomal fractions were boiled with 10 μ l SDS PAGE sample buffer (50 μ l Mercaptoethanole + 950 μ l 2X laemmli sample buffer) at 95 °C for 5 minutes

then two acrylamide gel cast were fixed into the box of electrophoresis in SDS PAGE buffer. After that 10 μ l exosomal fractions were loaded in the wells of the two acrylamide gels with 5 μ l marker for Coomassie Dyeing: Precision Plus Protein™ Dual Color Standards (Bio-Rad USA, lot # 100-22140) and 5 μ l marker for western blot: Precision Plus Protein™ Western C™ Standards (Bio-Rad USA, lot # 101-0376) then vertical electrophoresis was carried out at 100 V for 60 minutes.

1. Coomassei dyeing

The gel cast that specified for coomassei dyeing was immersed into Coomassie Brilliant Blue dye, and rock gently by the shaker at the room temperature for 30 minutes then was transfered to decoloring buffer with shaking gently by shaker for 15 minutes and discard the used buffer. Repeat the decoloration for 4 times then a photo for the gel was taken by ChemiDoc™ Touch imaging system.

2. Western blotting

Two filter papers, a nitrocellulose membrane (Bio-Rad, USA, cat # 162-0112) and the second gel cast that specified for western blotting were dipped in transfer buffer followed by transferring to the semi dry transfer machine for gel electrophoresis. After transferring, blocking step was carried out through submerging the membrane into 5% skim milk, and shake gently for 2 h then embed the blocked membrane into primary antibody (BLV2) (Ascites anti BLV gp51 ascites monoclonal antibody, 1.0 mg/ml, VMRD, Pullman WA 99163, USA, lot # P130627-003) diluted with 10 ml 1X PBST (Takara, Japan, lot # AG1P014) and shake gently for 1 h then wash the membrane by 1X PBST for 10 minutes 3 times. After that, embed the washed membrane into secondary antibody (Goat polyclonal antibody to mouse IgG, HRP conjugated, 2mg/ml, abcam ab6789 <http://www.abcam.com/goat-mouse-igg-hl-hrp-ab6789.html> lot # GR242773-1) diluted with 10 ml 1X PBST and shake gently for 1 h then wash the membrane by 1X PBST for 10 minutes 3 times.

Immerse the washed membrane in a mixture of 750 μ l solution A (Luminal Enhancer Solution) and 750 μ l solution B (Peroxide Solution) (ECL™ prime western blotting detection reagent, GL Healthcare, lot # 9672749) and cover the membrane with para film then incubate for 5 minutes and take a photography by ChemiDoc™ Touch imaging system.

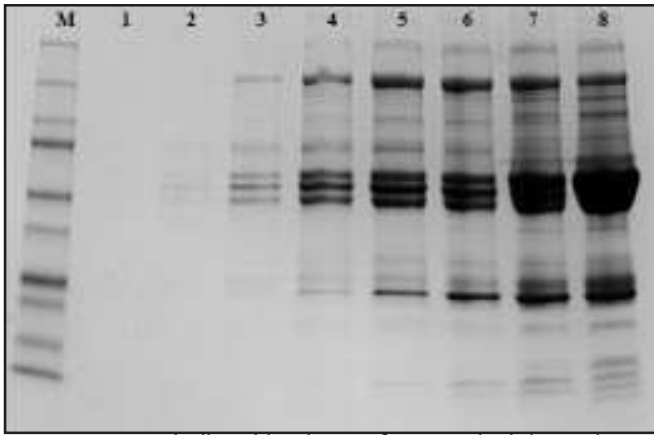


Fig 1. Coomassie brilliant blue dyeing after vertical gel electrophoresis of exosomal fractions. Proteins bands of different molecular weights were detected. M: is Coomassie marker or ladder and lanes from 1-8: are exosomal fractions.

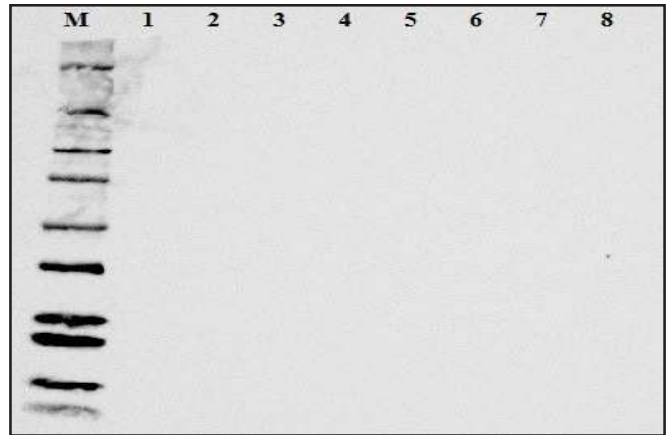


Fig 2. Immunoblotting for detection of BLV env protein showed that no bands at the target molecular weight 51 kD and all fractions appear to be negative for BLV env protein. M: is Western blot marker or ladder and lanes from 1-8: are exosomal fractions

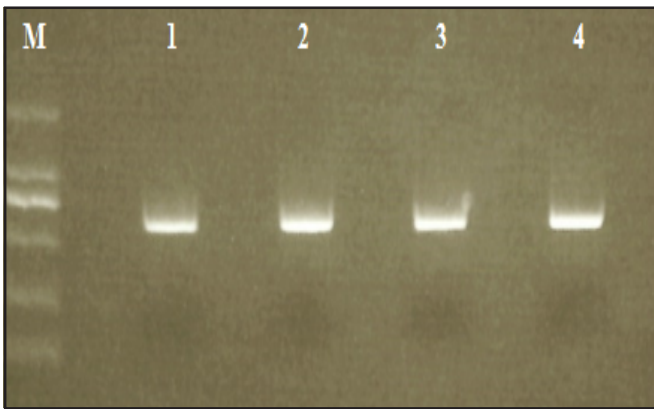


Fig 3. The results of gel electrophoresis after RT-PCR. Exosomal fractions from 4 samples showed +ve band at the target size (598bp). M is wide range DNA ladder and lanes are exosomal fractions.

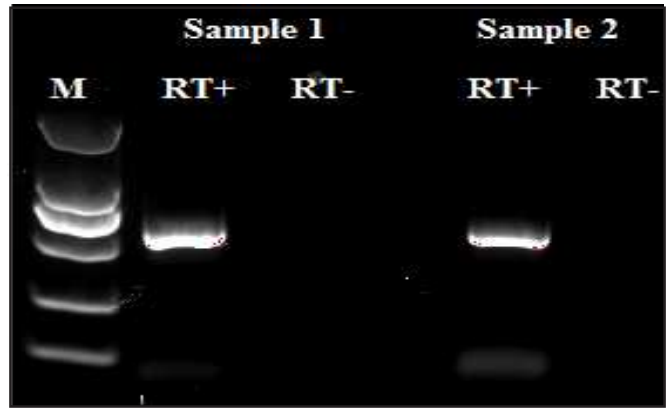


Fig 4. The results of gel electrophoresis after (RT+ Ve: RT - Ve) confirmation test, +Ve bands were detected at the target size (598 bp) at the RT+Ve lane. M: is wide range DNA ladder, RT +Ve lane: RT- PCR and RT - Ve lane: Normal PCR

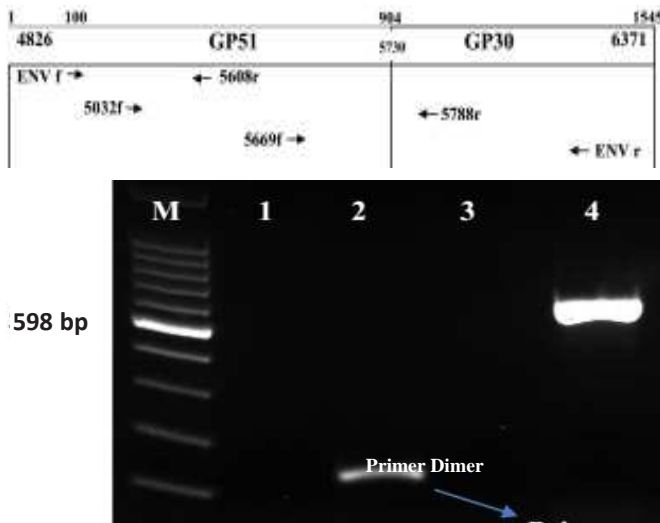


Fig 5. The results of gel electrophoresis after RT-PCR using different primer sets for BLV env gene (partial and full), A: BLV env precursor and its nucleotide position in the genome. B: Only partial BLV env gene primers 5032 F and 5608 R showed +ve band at the target size (598bp). M is DNA ladder, Lane 1: Primers 5032 f+5788 r, Lane 2: Primers Env f+ 5608 r Lane 3: Primers 5669 f+ Env r and Lane 4: Primers 5032 f+ 5608 r

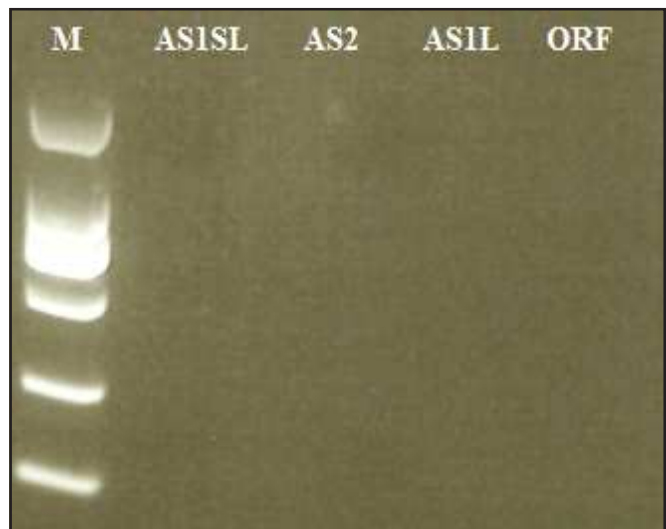


Fig 6. Results of RT-PCR using antisense primers. No bands were detected at target sizes. M is wide range DNA ladder, Lanes are antisense primers sets.

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      310      320      330      340      350      360      370      380      390      400      410
sample 1 Fr 6 GGAGCCAGGCCAATGCCACATATGATTTGGAGCCCGATGCCCTTATGTGGGGCAGATCCCTTCGACTGCCCCACTGGGACAAATGCCCTCCAGCCGATCAAGGATC
sample 1 Fr 7 .....
sample 1 Fr 8 .....
sample 2 Fr 6 .....
sample 2 Fr 7 .....
sample 2 Fr 8 .....
Sample 3 Fr 6 .....
Sample 3 Fr 7 .....
Sample 3 Fr 8 .....
Sample 4 Fr 6 .....
Sample 4 Fr 7 .....
Sample 4 Fr 8 .....

      420      430      440      450      460      470      480      490      500      510      520
sample 1 Fr 6 CTTTATGTCATATCAGATTTTATTCCTGGATCTCAACAATGTCATGGCAATTTCACTCTAACCTGGGAGATATGGGATATGATCCCTGATCACCTTTTCTTTAC
sample 1 Fr 7 .....
sample 1 Fr 8 .....
sample 2 Fr 6 .....
sample 2 Fr 7 .....
sample 2 Fr 8 .....
Sample 3 Fr 6 .....
Sample 3 Fr 7 .....
Sample 3 Fr 8 .....
Sample 4 Fr 6 .....
Sample 4 Fr 7 .....
Sample 4 Fr 8 .....

      530      540      550      560      570      580      590      600      610      620      630
sample 1 Fr 6 ATAAATCCCTGATCCCCCTCAACCAGACTTTCCCGASTTGAACAGTACCTGGTTCCCTCTGTTCAGATCAATGGGCCCTGCTTTTAAATCAACAGCAGGGCCCTCCCA
sample 1 Fr 7 .....
sample 1 Fr 8 .....
sample 2 Fr 6 .....
sample 2 Fr 7 .....
sample 2 Fr 8 .....
Sample 3 Fr 6 .....
Sample 3 Fr 7 .....
Sample 3 Fr 8 .....
Sample 4 Fr 6 .....
Sample 4 Fr 7 .....
Sample 4 Fr 8 .....

      640      650      660      670      680      690      700      710      720
sample 1 Fr 6 GACTGTCTATATGTGGGAACCTTCCCTCCCTGGGCCCCCAATATTANATATATACAAACCAATCTCCAGCTCTGGCCCGGCTCCGC
sample 1 Fr 7 .....
sample 1 Fr 8 .....
sample 2 Fr 6 .....
sample 2 Fr 7 .....
sample 2 Fr 8 .....
Sample 3 Fr 6 .....
Sample 3 Fr 7 .....
Sample 3 Fr 8 .....
Sample 4 Fr 6 .....
Sample 4 Fr 7 .....
Sample 4 Fr 8 .....

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Fig 7. Sequence analysis of exosomal fractions (6, 7 and 8) from four +ve RT-PCR samples. 100% nucleotide homology were found among all the examined fractions.

Extraction of exosomal RNA

BLV RNA was extracted from the exosomes for RT-PCR using NucleoSpin® RNA virus kit, (Macherey-Nagel, Germany) according to manufacture instruction supplied with the kits.

One-step RT-PCR

One step RT-PCR was carried out for detection of the exosomal RNA using Qiagen one step RT-PCR kit, Cat. # 210212, lot. # 151010159 according to the manufacture instruction and the target RNA product was detected in 25µl mix consists of 13 µl RNase free water, 1.5 µl Forward primer, 1.5 µl Reverse primer, 5 µl 5 X Buffer, 1 µl dNTP mixture, 0.1 µl enzyme mix and 2 µl RNA template.

The reaction was carried out using thermal-cycler (Life ECO, China) in the following cycles with a small modification acc. to the primers used (Table 1): reverse transcription at 50 °C for 30 minutes for one cycle then initial denaturation at 95 °C for 15 minutes for one cycle then 35 cycles consisting of denaturation at 95 °C for 10 seconds then annealing at 60 °C for 10 seconds then extension at 72 °C for 15 seconds then one cycle final extension at 72 °C for 10 minutes.

PCR amplification and gel electrophoresis (cDNA)

After RT-PCR and obtaining positive bands for the resulted cDNA, PCR amplification and gel electrophoresis were carried out on the reverse transcribed positive RNA samples to obtain DNA gel bands for sequential analysis using forward primer (env 5032) and reverse primer (env 5608) as described by Asfaw *et al* (2005) (Table 1). The target product was detected in 20µl mix consists of 15.1 µl Distilled water, 0.1 µl Forward primer env 5032, 0.1 µl Reverse primer env 5608, 2 µl 10 X Ex Taq Buffer, 1.6 µl dNTP mixture, 0.1 µl Ex Taq HS and 1 µl DNA template

The amplification reaction was carried out using thermal-cycler as follow: Initial denaturation at 94 °C for 9 minutes for one cycle then 40 cycles consisting of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 30 seconds, extension at 72 °C for 1 minute then one cycle final extension at 72 °C for 4 minutes then holding at 4 °C “.

The PCR products were placed on a gel plate prepared using Tris EDTA buffer containing 1% sea Kem® agarose (cat # 50.000, lot # 0000385106), stained by GelRed stain (GelRed™ Nuclis Acid Gel Stain. 10.000X in DMSO cat # 41002) then electrophoresed

Table 3. RNA concentration and quality using NanoDrop of the exosomal fraction after RNA extraction.

Sample ID	RNA Conc. ng/ul	260/230
Fraction 6 sample 1	423	3
Fraction 7 sample 1	362.8	3.11
Fraction 8 sample 1	288.8	2.85
Fraction 6 sample 2	457.4	3.26
Fraction 7 sample 2	345.3	3.2
Fraction 8 sample 2	287.3	3.18
Fraction 6 sample 3	249	3.02
Fraction 7 sample 3	231.1	3.05
Fraction 8 sample 3	212.7	3.11
Fraction 6 sample 4	240.4	3.02
Fraction 7 sample 4	229.2	3.16
Fraction 8 sample 4	264.8	2.98

at 100 V for 30 minutes and finally visualized by ultraviolet light after using a DNA ladder.

DNA purification and extraction from the gel

The DNA that detected in the target band after PCR amplification and gel electrophoresis was extracted and purified from the gel for sequencing using QIAquick® Gel Extraction Kit (250), QIAGEN acc. to manufacturer protocol, then the resulted purified DNA products were stored at -20 till used.

Sequential analysis

Sequenc analysis was carried out to detect the nucleotide composition of the purified DNA products and it was carried out in both directions forward (*env* 5032 f) and reverse (*env* 5608 r) (Table 1) using Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK, lot #. 1111211) acc. to manufacturer's instruction and then analyzed with an Applied Biosystems 3730 DNA Analyzer.

The reaction was performed in 20 µl reaction volumes containing 10.2 µl Distilled water, 0.3 µl env primer, 4.5 µl Diluted BigDye and 5 µl DNA template.

The reaction was carried out using thermal-cycler using the following cycles: PCR activation at 96 °C for 1 minute for one cycle, then 25 cycles consists of denaturation at 96 °C for 10 seconds then annealing at 50 °C for 5 seconds then extension at 60 °C for 4 minutes and then holding at 4 °C.

Results

Serological detection of BLV infection using ELISA

Since there is no vaccination against BLV infection, a presence of antibodies was an accurate indicator of natural infection. ELISA is a routine technique for detection of BLV specific antibodies in laboratory of animal infectious disease and prevention (AIDP) university of Miyazaki, Japan, and the test was interpreted as the samples with S/P value below 0.30 considered negative and more than 0.30 considered positive acc. to this equation:

$$S/P \text{ Value} = \{S (P) - S (N)\} / \{PC (P) - PC (N)\}$$

PC (P): Average absorbance of the positive control in the antigen positive wells.

PC (N): Average absorbance of the positive control in the antigen negative wells.

S (P): Absorbance of the sample in the antigen positive well.

S (N): Absorbance of the sample in the antigen negative well.

Finally, 18 fresh ELISA positive samples were selected for exosomes isolation (Table 2).

Detection of the BLV proviral load using real-time PCR

From the whole blood of ELISA positive samples, BLV proviral DNA was extracted and its concentration was measured (ng/ul) using NanoDrop 8000 spectrophotometer and adjusted by dilution to be (50 ng/ul) then subjected to real-time PCR for detection of BLV proviral load (Copy/50 ng) then the results were recorded.

Out of 18 samples, 16 samples were of very high proviral load more than 2000 Copies/50 ng (samples from 1 to 16) and two samples with very low proviral load less than 2 Copies/50 ng (Samples 17 and 18) (Table 2).

Detection of exosomal BLV proteins

Coomassie Brilliant Blue dyeing

After exosomes isolation, the exosomal fractions of one gel cast were vertically electrophorized then stained by Coomassie brilliant blue dye method for screening the presence of any kind of protein inside the exosomes and the results showed that different proteins bands with different molecular weights were detected inside the exosomal fractions as shown in Fig1.

Western blot technique

After vertical electrophoresis of the second gel cast, the exosomal fractions were subjected to western blot for investigation the presence or absence of BLV env protein using (BLV2) as primary antibody and Goat polyclonal antibody to mouse IgG, HRP conjugated antibody as secondary antibody. The results showed that all the tested fractions from all samples showed no bands at target molecular weight (51 kD) of gp51 env protein as shown in Fig 2.

Detection of exosomal BLV RNA using RT-PCR

The exosomal BLV RNA was extracted from the fractions showing high exosomes population from each sample (Fraction 6, 7 and 8) and its concentration and quality were detected using NanoDrop 8000 spectrophotometer. The results indicated that only 16 fractions from four samples exhibited RNA (Table 3).

The extracted RNA was used for RT-PCR. The results of gel electrophoresis after RT-PCR showed that 12 fractions from 4 samples shows positive bands at the target size (598 bp) using partial BLV env gene primers 5032 F and 5608 R as shown in (Fig 3).

For confirmation of the results of RT-PCR and to exclude the contamination with BLV proviral DNA we carried out (RT +ve which means RT-PCR with reverse transcription & RT-ve which means PCR without reverse transcription) confirmation test using partial BLV env gene primers 5032 F and 5608 R (598 bp). The results showed +ve band in RT +ve lane only which confirm the presence of BLV RNA and absence the possibilities of contamination with BLV proviral DNA as in (Fig 4).

To identify the position of the detected RNA on the BLV env precursor, several primer sets were used targeting different parts of BLV env gene either partially or fully (Table 1) and RT-PCR was carried out. The results showed +ve bands only using partial BLV env gene primers 5032 F and 5608 R (598 bp) which indicated that the detected RNA is partial gp51 env gene as shown in (Fig 5 A and B).

BLV antisense transcripts were investigated by carrying out RT-PCR using four sets of antisense primers on the previously positive RNA samples (Table 1). The results showed that no bands were detected at target sizes (AS1SL 600 bp, AS2 400 bp, AS1L 2200 bp and anti ORF 264bp) which ensure absence of BLV antisense transcripts in tested exosomes as shown in (Fig 6).

Sequence analysis of the exosomal partial BLV RNA

The purified DNA after PCR amplification was succumbed to sequential analysis in both directions using BLV env gene primers 5032 F and 5608 R. After direct sequencing of the 444-bp PCR products, analysis carried out of 423-nucleotide sequences corresponding to part of the BLV env gp51-encoding gene (from nucleotide s 5126 to 5548 of the full length BLV genome and from nucleotide s 301 to 723 of whole env gene).

The results of sequence analysis showed that 100% nucleotide homology were present which indicate that only one genotype circulate in the infected animals as shown in Fig 7.

Discussion

In the context of RNA viral infection, several studies have observed that naive recipient cells exposed to RNA viruses derived exosomes harbor and deliver many regulatory factors including viral RNA, miRNA and proteins to the neighboring cells affecting on their physiological performance (Chahar *et al*, 2015). Because of the BLV pathogenesis is not fully clear and the role of exosomes in BLV infections is poorly understood, our objective was to identify how exosomes secreted from BLV infected cells might contribute to BLV life cycle hypothesizing that exosomes can transfer BLV proteins and RNA from infected to uninfected cells.

In the present study, we used size exclusion chromatography method after filtration of the plasma samples using 0.22-mm filter for isolation of exosomes to obtain a more uniform vesicle size of exosomes and superior recovery of their components (proteins and RNA) and also this help in removing contaminating cell debris and other larger microvesicles (Taylor *et al*, 2011; Jaworski *et al*, 2014; Nordin *et al*, 2015; Welton *et al*, 2015).

Results of Coomassie brilliant blue dye method indicated the presence of different proteins of different molecular weight within all the exosomes which is indicated by presence different protein bands at different sizes (Fig 1), this finding harmonized with the results of (Subra *et al*, 2010; Chahar *et al*, 2015) who confirmed that protein composition in all exosomes have been characterized by presence of a wide variety of proteins and also agreed with (Mathivanan *et al*, 2012) who said that exosomes contain about 4,563 types of proteins.

Results of western blotting in Fig 2 indicated that absence of BLV env protein in the all tested exosomal fractions, this result was in contrary to the results of

(Yamada *et al*, 2013) who detected BLV structural proteins, gp51 (Env) and p24 (Gag) in purified bovine milk exosomes, this disagreement may be due to the difference in the type of samples and collection timing which may influence the cellular system for sorting exosomal contents.

RT-PCR results showed that partial BLV RNA was detected in exosomes isolated from four samples out of 18 samples suggesting that exosomes might play a role in the viral life cycle through transfer the RNA from infected to non-infected cells. These results agreed with Narayanan *et al*, (2013) who showed that the HTLV-1 (which closely related to BLV) infected cells secrete exosomes that contain short viral RNAs that can serve as vehicles to deliver functional HTLV-1 mRNA to recipient cells. Vojtech *et al*, 2014 and Bukong *et al*, 2014 also reported that exosomes derived from RNA viruses infected cells can carry various RNAs.

Regarding to the BLV antisense transcripts, our results showed that absence of BLV antisense transcripts in all tested exosomes as in Fig 6, this result is in disagreement with the results of (Durkin *et al*, 2016) who detected BLV antisense transcripts in L267 tumor B cell line samples, and this may be due to the change in samples types used for examination.

Sequence analysis of the detected exosomal RNA showed that 100% nucleotide homology were found which indicate the probability of circulation of the same BLV strain in all the BLV infected animals examined Fig 7.

Conclusion

From the results of current study we can conclude that the exosomes may be incriminated in contribution to BLV pathogenesis or life cycle through transfer of BLV genetic material (RNA) from infected to non-infected cells but further studies must be carried out to know more details about the nature and the pathological effect of the transferred RNA within the recipient cells.

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