The Journal of Veterinary Medical Science

FULL PAPER

Virology

Detection and molecular characterization of bovine leukemia virus in beef cattle presented for slaughter in Egypt

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ABSTRACT. Bovine leukemia virus (BLV) is the etiological agent of enzootic bovine leukosis, the most common neoplastic disease of cattle worldwide and a serious problem for the cattle industry. Previous studies have shown the molecular prevalence of BLV and the coexistence of BLV genotype-1 and -4 in Egyptian dairy cattle; however, the molecular characteristics of BLV in Egyptian beef cattle are unknown. Therefore, we collected blood samples of 168 beef cattle from slaughterhouses in three governorates in Egypt. Based on BLV-CoCoMo-qPCR-2 targeting long terminal repeats and nested PCR targeting the *env-gp51* gene, the BLV provirus infection rates were found to be 47/168 (28.0%) and 42/168 (25.0%), respectively. Phylogenetic analysis based on 501 bp of the BLV *env*-gp51 gene from 42 BLV isolates revealed that at least six distinctive strains (b, e, f, g, x, and z) were prevalent in cattle across the examined regions. Furthermore, phylogenetic analysis of the 420 bp sequence of the BLV *env*-gp51 region of the six strains against 11 known genotypes showed that the strains b, e, f, and g were clustered into genotype-1, and strains x and z were clustered into genotype-4. Our results also indicated that strains b and x exist in both dairy and beef cattle in Egypt. The present study is the first to detect and genotype BLV among beef cattle in Egypt.

KEY WORDS: beef cattle, bovine leukemia virus, Egypt, genotyping, prevalence

J. Vet. Med. Sci.
82(11): 1676–1684, 2020
doi: 10.1292/jvms.20-0477

Received: 12 August 2020
Accepted: 4 September 2020
Advanced Epub:
20 October 2020

Bovine leukemia virus (BLV) is a member of the *Retroviridae* family. It belongs to the genus *Deltaretrovirus* and is closely related to human T-cell leukemia virus types 1 [3]. BLV naturally affects domestic cattle and water buffaloes and induces enzootic bovine leukosis (EBL), which is the most common neoplastic disease of cattle worldwide [3, 27]. Following infection with BLV, only a small proportion of infected individuals develop lymphomas and/or show B-lymphocyte proliferation. However, the majority of infected cows do not show any clinical symptoms or changes in lymphocytic count, but are asymptomatic carriers of the virus [3]. Digestive disturbance, weight loss, weakness, reduced meat and milk production, loss of appetite, and enlarged lymph nodes are the main characteristics of lymphoma stage during BLV infection in cattle [3, 36]. Lymphomas caused by BLV are typically seen predominantly in animals over three years old. However, recently EBL has been reported in cattle younger than two years old [18, 30]. Vertical transmission of BLV can occur via in utero infection and ingestion of infected milk and colostrum [12], and infected calves have the potential to develop EBL at a younger age. The loss of young successors or fattening cattle can cause major economic damage to farms [18]. Furthermore, infected calves can spread the virus within farms over a longer period of time than cows infected at an older age, thus resulting in large economic losses. Importantly, young fattening calves under two years old are often sent to the slaughterhouse for meat production.

According to the FAOSTAT database, in 2018, Egypt's cattle herd was estimated at 4.5 million heads (www.fao.org/faostat/

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en/#data/QA). Egypt's entire livestock herd, including both cattle and buffalo, is maintained primarily for dairy production, with meat production of secondary importance. All cattle herds consist of either mixed local breeds or imported dairy cattle; females are kept for milk production, while male animals and infertile females are fattened for meat [43]. The Egyptian beef sector is highly heterogeneous, comprising of intensive, semi-intensive and extensive systems. Intensive beef production systems provide the best quality meat, while the semi-intensive systems produce the majority of beef for the country. In contrast, extensive systems (small holders) provide surplus young calves, bulls and unproductive females for slaughtering. Beef animals are sold a live, either in livestock markets or to butchers and/or directly to slaughter houses in large cities [2]. Egypt remains dependent on imported live beef cattle and frozen imported beef due to the shortage of meat production. Brazil, Australia, Sudan, and Ethiopia are Egypt's main suppliers for live cattle for immediate slaughter [43].

In Egypt, EBL was first reported in the Assiut governorate by Zaghawa *et al.* in 1997 [48]. They investigated cattle from a Holstein-Friesian dairy herd (n=440) imported from Minnesota, USA, and some of them were suffering from enlargement of superficial lymph nodes. Serological screening using enzyme-linked immunosorbent assays (ELISA) revealed a seroprevalence of BLV antibodies of 37.7% in cattle under two years old and 72.8% in animals more than two years old [48]. This diagnosis was confirmed through the detection of BLV proviral DNA, which indicates the retroviral genome has integrated into the host genome, in 15 individuals. The World Organization for Animal Health (OIE) considers that Egypt has been free from BLV since 1997 [32]. However, a number of serological studies have detected BLV infections among Egyptian cattle in different regions such as, Kafr El Sheikh, Alexandria and Menofia; with infection rate of 15.83% [20], and in Kafr El Sheikh, Qalyubia and Menofia was 20.8% in Egyptian dairy cows [38]. Our recent study investigating the distribution of the BLV provirus, showed that BLV infections were prevalent in 21.5% (58 out of 270 head) of Egyptian dairy cattle in five provinces located in the northern, central, and southern parts of Egypt [11]. In contrast, to date, there have been no reports regarding the prevalence of BLV among beef cattle in Egypt. Some studies have estimated this in other countries, for example, in USA, seroprevalence of BLV among beef cattle presented for slaughter was 33.6% [6]. In addition, a nationwide survey of BLV infections among cattle in Japan from 2009–2011 using ELISA reported that 28.7% of beef breeding cattle tested positive for BLV [28]. The prevalence of BLV infections among Zambian beef cattle was found to be 2.1% of the pooled samples [33]. This demonstrates the need to determine the prevalence and distribution of BLV provirus among Egyptian beef cattle.

The genomic structure of BLV is composed of structural and enzymatic regions that encode genes, namely *gag*, *pro*, *pol*, and *env*; as well as the pX region, which encodes two regulatory genes (*tax* and *rex*) and two accessory genes (*R3* and *G4*), and is flanked by two identical long terminal repeats (LTRs) at the 5′ and 3′ termini [3]. The BLV *env* gene is transcribed as an envelope glycoprotein (Env) protein complex that is composed of surface (gp51) and transmembrane (gp30) proteins [10]. BLV infection is mediated by interactions between gp51 and the recently identified cellular receptor cationic amino acid transporter 1 [5]. The glycoprotein gp51 contains three conformational epitopes (F, G, and H) in its N terminus, which are recognized by neutralizing antibodies [8, 10]. The gp51 C terminus also contains 4 linear epitopes (A, B, D, and E) [7]. There is also evidence that the N-terminal half of the mature gp51 glycoprotein has important roles in virus infectivity and syncytium formation [15]. Therefore, the gp51 region is widely used for BLV genotyping studies. Based on phylogenetic analysis of the *env-gp51* sequence region of BLV, at least 11 BLV genotypes have been reported in different countries globally [9, 22–24, 33–35, 37, 46]. Three BLV genotypes, namely genotypes-1, -4, and -6 are the most commonly detected around the world [36].

Previous studies have demonstrated the molecular prevalence of BLV and the coexistence of BLV genotypes-1 and -4 in Egyptian dairy cattle [11]. However, the molecular characteristics of BLV among beef cattle in Egypt remain unknown. In this study, we investigated the prevalence of BLV infections among beef cattle in three different regions of Egypt using real-time polymerase chain reaction (PCR) targeting the BLV LTRs, and nested PCR targeting the BLV *env-gp51* gene. We also performed a phylogenetic analysis of BLV strains to characterize the BLV genotypes circulating in beef cattle in the examined regions of Egypt.

MATERIALS AND METHODS

Ethical approval

All animals were handled by the regulation of the Animal Ethics Committee at the Faculty of Veterinary Medicine, South Valley University, Qena, Egypt, and by the regulation of RIKEN, Japan in strict accordance with good animal practice following the guidelines of RIKEN. The study was reviewed and approved by Research Code of Ethics (RCOE-SVU) at the South Valley University, and by the RIKEN Animal Experiments Committee (approval number H29-2-104).

Sample population

A total of 168 healthy male beef cattle presented for slaughter were randomly sampled from three abattoirs in different localities in Egypt in 2018. The cattle were of mixed breed and were of various ages ranging from 1.5 to 3 years old and weighed between 300–500 kg. They were divided into three groups according to the area of collection and type of cattle farming system: Group 1-animals sampled from a Cairo governorate slaughter house in northern Egypt and kept in an intensive farming system (more than 200 heads) (n=53); Group 2-animals sampled from a Fayoum governorate slaughter house in central Egypt and kept in an individual small holder farming system (1–20 heads) (n=10); and Group 3-animals sampled from a Qena governorate slaughter house in southern Egypt and kept in an individual small holder farming system (n=105). None of the carcasses of the sampled animals showed any postmortem pathological lesions and were judged by veterinarians as fit for human consumption. The numbers and characteristics of the groups of sampled cattle and the locations of the abattoir sites are shown in Table 1.

Table 1. Prevalence of bovine leukemia virus infection examined using CoCoMo-qPCR-2 targeting long terminal repeats (LTRs) and nested PCR targeting *env*-gp51, among beef cattle in Egypt

Province	Slaughter house	Farming system [a)]	LTR	*env*-gp51
			Positive No./ Tested No. (%)	Positive No./ Tested No. (%)
Cairo	Bsateen	Intensive [b)]	41/53 (77.4%)	39/53 (73.5%)
Fayoum	Fayoum	Small holder [c)]	1/10 (10.0%)	1/10 (10.0%)
Qena	Al salhia	Small holder	5/105 (4.8%)	2/105 (1.9%)
Total			47/168 (28.0%)	42/168 (25.0%)

a) The tested cattle were males, mixed breed, 1.5–3 years old, and weight 300–500 kg. b) Intensive farming (more than 200 heads). c) Small holder farming (1–20 heads).

Blood sampling and DNA extraction from blood samples

Whole blood samples were collected from animals which were physically constrained using the tail vein puncture procedure, and were stored in a glass tube with K_2 EDTA anticoagulant. Blood samples were transported immediately into the laboratory and kept at 4°C until DNA extraction was carried out. Genomic DNA was extracted from the K_2 EDTA anticoagulated whole-blood samples using the Wizard Genomic DNA Purification Kit (Promega Corporation; Madison, WI, USA) according to the manufacturer instructions. The extracted DNA was kept at −20°C prior to analysis.

Detection of BLV provirus using real time-PCR

The prevalence of BLV infection was confirmed by BLV-CoCoMo-qPCR-2 (RIKEN Genesis, Yokohama, Japan) [39]. In brief, the genomic DNA was normalized to 30 *ng*/*µl* using a NanoDrop One Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), then 150 *n*g of genomic DNA was added to each quantitative PCR. BLV LTR regions were amplified in a reaction mixture containing THUNDERBIRD Probe qPCR Mix (Toyobo; Tokyo, Japan) using the degenerate primer pair of BLV CoCoMo primers (CoCoMo FRW and CoCoMo REV) and a 6-carboxyfluorescein (FAM)-labeled LTR probe. To normalize the viral genomic DNA level within the host cellular genome, a single copy of *bovine leukocyte antigen (BoLA)-DRA* gene was amplified using the primers DRA-FW and DRA-RW, and FAM- labeled DRA probes. Different dilutions of the standard plasmids were used to generate the correct copy numbers of the *BoLA-DRA* gene (for genome reference) and BLV-LTR (for the BLV provirus). Thermal cycling was carried out using a Roche Light Cycler® 480 Instrument II plate-based real-time PCR amplification system (Roche, Basel, Switzerland), in which denaturation took place for 1 min at 95°C, and was followed by 45 cycles of 15 sec at 95°C, and then 1 min at 60°C. The provirus load (PVL) was calculated using the following equation: number of BLV-LTR copies/number of *BoLA-DRA* copies) $\times 10^5$ cells. Finally, the positive samples were detected, based on the criteria of ≥1 copy of provirus in 10^5 cells.

Detection of BLV env-gp51 gene fragments using nested PCR

BLV infections were detected using nested PCR, through partial amplification of the BLV *env-gp51* gene using PrimeSTAR GXL DNA Polymerase (Takara Bio Inc., Kusatsu, Japan) according to the manufacturer instructions. First, external PCR primers were used, followed by internal PCR primers, as described previously [4, 25, 35]. Briefly, the reaction mixture contained 13.5 *µl* (external PCR) and 14.5 *µl* (internal PCR) of distilled water, 5 *µl* of 5 ×PS GXL Buffer, 2 *µl* of 2.5 *µ*M dNTP mix, 0.5 *µl* of PrimeSTAR GXL, and 1 *µl* of each primer (10 *µ*M). The PCR amplification conditions were as follows: 98°C for 2 min, followed by 30 cycles of denaturation at 98°C for 15 sec, annealing at 60°C for 20 sec, and extension at 68°C for 1 min. The external primers resulted in amplification of a 913 bp DNA fragment, and the internal primers amplified a 598 bp fragment of the gp51 region of the *env* gene.

Nucleotide sequencing and phylogenetic analysis of BLV env-gp51 gene fragments

Following amplification of a 598 bp fragment of the BLV *env*-gp51 gene as described above, the positive second-round PCR products were purified using Exo-SAP IT (USB Corp., Cleveland, OH, USA) and sequenced on an ABI3730xl DNA Analyzer using an ABI PRISM Big Dye Terminator v 3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), using the same forward and reverse primers for the second nested PCR.

In order to detect genetic variation, we constructed a maximum likelihood (ML) phylogenetic tree. This was based on 501 bp of the BLV *env*-gp51 gene, corresponding to nucleotide positions 5084–5584 of the whole BLV genomic sequence (GenBank accession number LC164083) [26], based on the 42 isolates of BLV from beef cattle in this study, and 50 isolates from dairy cattle in Egypt. Analysis was performed using the software MEGA7 [19]; the Kimura-2 (K2) model was chosen, as it was determined to be the best model for nucleotide substitution [16].

For BLV genotyping, six typical strains out of 42 were selected for construction of another ML phylogenetic tree. This was based on 420 bp of the *env* gene, corresponding to nucleotide positions 5126–5545 of the whole BLV genomic sequence, together with 55 BLV *env*-gp51 gene sequences from GenBank. These included sequences from European, Asian and African countries including Egypt, which were representative of the 11 known BLV genotypes. The Kimura-2 parameter model plus gamma distribution (K2+G) was chosen for this analysis as it was deemed to best model for nucleotide substitution, and was performed using the software MEGA7 [19].

RESULTS

BLV infection rate among Egyptian beef cattle

To investigate the spread of BLV infections in Egypt, we collected blood samples from 168 male beef cattle, from three abattoirs in different governorates in Egypt: Cairo, Fayoum, and Qena. Genomic DNA isolated from these samples was tested for the presence of BLV infection using nested PCR targeting the BLV *env-gp51* gene, and BLV-CoCoMo-qPCR-2 targeting LTRs (Table 1). Using BLV-CoCoMo-qPCR-2, 47 out of 168 samples (28.0%) were identified as positive for BLV provirus, whereas 42 of the samples (25.0%) were found to be positive using nested PCR. Based on BLV-CoCoMo-qPCR-2, in the Cairo, Fayoum, and Qena governorates, the prevalence of BLV infection was 41/53 (77.4%), 1/10 (10.0%) and 5/105 (4.8%), respectively. Whereas, based on the nested PCR results, BLV *env-gp 51* gene fragment amplification was found in 39/53 (73.5%), 1/10 (10.0%) and 2/105 (1.9%) of samples, in the Cairo, Fayoum and Qena governorates, respectively.

Identification of the six genetically distinct strains circulating in Egyptian beef cattle using phylogenetic analysis based on the 501 bp sequence of gp51-env region

To analyze genetic variability among the BLV strains isolated from Egyptian beef cattle, we amplified 42 BLV isolates, which were identified as containing BLV using both BLV-CoCoMo-qPCR-2 and nested PCR. After direct sequencing, 501 bp sequences corresponding to nucleotide positions 5084–5584 of the full-length BLV proviral genome of the reference strain; pBLV-FLK complete genome (GenBank accession number LC164083) [26]; were used to construct an ML phylogenetic tree (Fig. 1). In the ML phylogenetic tree, all 42 BLV strains isolated from the beef cattle aligned with the 50 isolates detected from Egyptian dairy cattle in our previous study [11];. The tree demonstrated the presence of six genetically different strains (b, e, f, g, x, and z). Notably, two of these strains, b and x, were identical to those isolated from Egyptian dairy cattle in Egypt. However, the four other strains, e, f, g, and z, had different sequences from those identified in the dairy cattle.

Nucleotide sequence analysis of the six genetically distinct strains circulating in Egyptian beef cattle, and four strains from Egyptian dairy cattle

Nucleotide sequences of the 12 selected typical BLV isolates representing the six genetically distinct strains (b, e, f, g, x, and z) circulating in Egyptian beef cattle, and the six genetically distinct strains (a, b, c, d, x, and y) that were previously isolated from Egyptian dairy cattle were aligned with that of the reference strain (Fig. 2). The level of similarity of the 501 bp sequence of the BLV *env*-gp51 region among the 12 selected typical BLV isolates ranged from 96.7–100% (data not shown). The sequence of the a strain previously isolated had 100% similarity with that of the reference strain. The two sequences of the b strain exhibited two nucleotide substitutions, one at nt 447 and the other at nt 609. In our previous study of dairy cattle, we only found one sequence of the c strain, which had three nucleotide substitutions (at nt 336, nt 447 and nt 609). Similarly, we previously detected one sequence of the d strain, which exhibited three nucleotide substitutions (at nt 296, nt 447 and nt 609). The 14 sequences of the e strain exhibited three nucleotide substitutions (at nt 447, nt 472 and nt 609). The two sequences of the f strain exhibited three nucleotide substitutions (at nt 447, nt 555 and nt 609). The single sequence of the g strain exhibited six nucleotide substitutions (at nt 375, nt 447, nt 525, nt 555, nt 559 and nt 609). We found 15 nucleotide substitutions (at nt 270, nt 362, nt 375, nt 408, nt 447, nt, 483, nt 525, nt 555, nt 559, nt 588, nt 591, nt 609, nt 615, nt 697 and nt 717), both in the 38 sequences of the x strain identified in dairy cattle in the previous study, and the 20 sequences of the x strain identified in the beef cattle. We found 16 nucleotide substitutions (at nt 270, nt 362, nt 375, nt 408, nt 447, nt 483, nt 525, nt 555, nt 559, nt 588, nt 591, nt 609, nt 615, nt 697, nt 715 and nt 717) in the sequences of the three y strains identified in the previous study, and 16 nucleotide substitutions (at nt 270, nt 362, nt 375, nt 408, nt 447, nt 483, nt 525, nt 555, nt 558, nt 559, nt 588, nt 590, nt 609, nt 615, nt 697 and nt 717) in the sequence of z strain identified in the beef cattle.

Phylogenetic analysis of the 420 bp sequence of the BLV env-gp51 region of the six genetically distinct strains circulating in Egyptian beef cattle and the known strains from different geographic locations worldwide

To demonstrate which BLV genotypes are circulating among Egyptian beef cattle, an ML phylogenetic tree was constructed. This was based on the 420 bp sequence of the BLV *env*-gp51 region, corresponding to nucleotide positions 5126–5545 of the whole BLV genomic sequence, using the 12 selected typical BLV isolates that represent the six strains isolated from Egyptian beef cattle and the six strains that were previously isolated from Egyptian dairy cattle, together with the sequences of 53 references (Fig. 3). The ML phylogenetic tree indicated that the isolated BLV strains circulating among Egyptian beef cattle belong to genotypes-1 and -4, and are circulating in different regions of Egypt. Notably, the four strains b, e, f, and g belong to genotype-1 and the other two strains, x, and z belong to genotype-4. For example, isolate EGY.QEN 40 that belongs to genotype-1 is representative of four isolates of strain b (Fig. 1) and is identical to Egypt-QB1.1, KU233533/Thailand and KP201481/Korea. However, isolate EGY.CAI 306 is representative of 14 isolates of strain e (Fig. 1) and shares a high similarity with EF065653/Japan. Isolate EGY.CAI 275, which is representative of two isolates of strain f (Fig. 1) and isolate EGY.CAI 270, which represents strain g (Fig. 1) are closely related to each other but less similar to other isolates of genotype-1. Furthermore, isolate EGY.CAI 258 is representative of 20 isolates of strain x (Fig. 1) and typical to Egypt-DZ1.6, and isolate EGY.CAI 271, represents strain z (Fig. 1), belongs to genotype-4. Both strains share 97% similarity and are closely related to AF503581/Belgium.

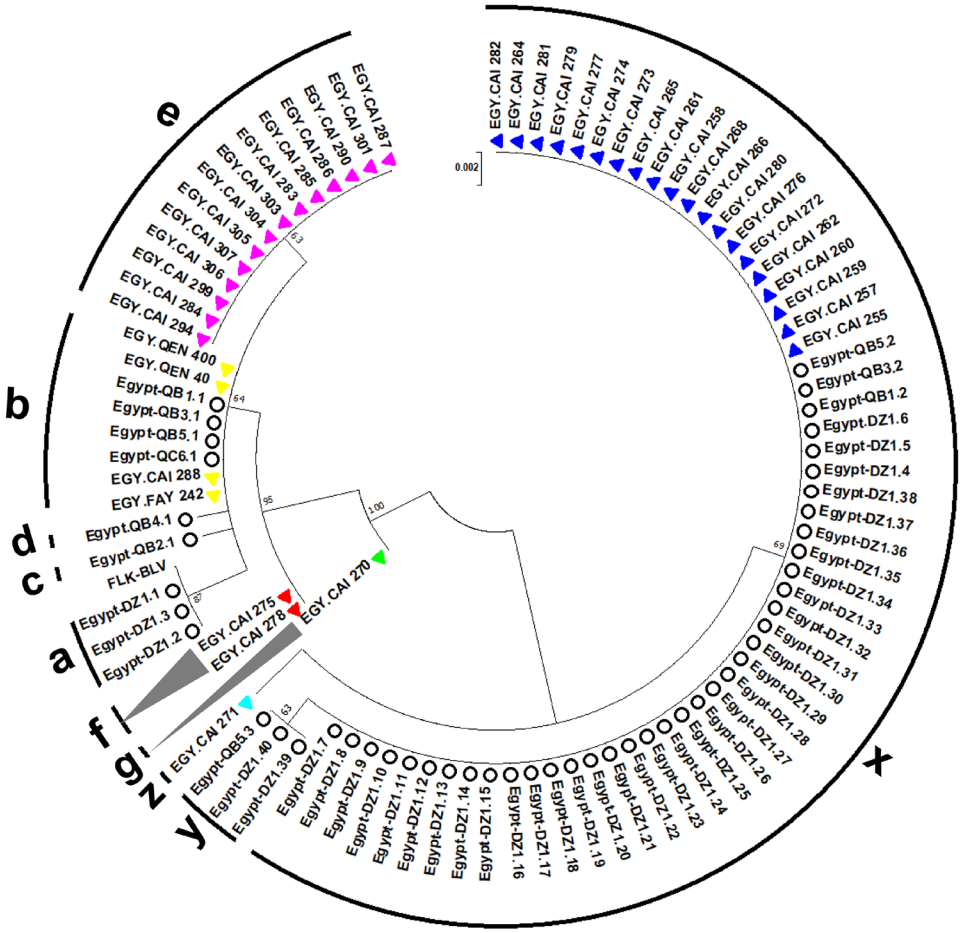


Fig. 1. Maximum likelihood (ML) phylogenetic tree based on 501 bp nucleotides of the bovine leukemia virus (BLV) *env*-gp51 gene from 42 BLV isolates, which represent the six genetically distinct strains circulating in beef cattle in Egypt (filled triangles), together with 50 isolates that were previously detected among dairy cattle in Egypt (opened circles). This tree was constructed for sequences of 42 BLV isolates detected in different three geographic locations in Egypt, including four isolates of "b" (yellow filled triangles), 14 isolates of "e" (pink filled triangles), two isolates of "f" (red filled triangles), one isolate of "g" (Green filled triangle), one isolate of "z" (Fluorescent filled triangle), 20 isolates of "x" (blue filled triangles), 50 isolates detected in our previous study of dairy cattle in Egypt (opened circles) and the reference strain: pBLV-FLK complete genome (GenBank accession number LC164083). Isolate labels are indicated by abbreviations of the country and city names (EGY. for Egypt, CAI, FAY, and QEN for Cairo, Fayoum, and Qena, respectively) along with sample ID. The GenBank accession numbers of the 42 BLV isolates (EGY.QEN40, EGY.QEN400, EGY.FAY242, EGY.CAI288, EGY.CAI306, EGY.CAI307, EGY.CAI305, EGY.CAI304, EGY.CAI303, EGY.CAI283, EGY.CAI284, EGY.CAI285, EGY.CAI286, EGY.CAI290, EGY.CAI294, EGY.CAI299, EGY.CAI301, EGY.CAI287, EGY.CAI275, EGY.CAI278, EGY.CAI270, EGY.CAI255, EGY.CAI257, EGY.CAI259, EGY.CAI260, EGY.CAI262, EGY.CAI272, EGY.CAI276, EGY.CAI280, EGY.CAI266, EGY.CAI268, EGY.CAI258, EGY.CAI261, EGY.CAI265, EGY.CAI273, EGY.CAI274, EGY.CAI277, EGY.CAI279, EGY.CAI281, EGY.CAI282, EGY.CAI264, EGY.CAI271) are LC553737-LC553778, respectively. Sequences of mutants were repeated in three independent replicates. The bar in the upper portion of the figure denotes distance.

DISCUSSION

This study provides the first report of the prevalence and molecular characteristics of strains of BLV among beef cattle in Egypt. Our group previously investigated the prevalence of BLV infections among Egyptian dairy cattle. However, until now the BLV infection rate among beef cattle in Egypt was unknown. In this study, we first demonstrated the BLV infection rate among beef cattle of mixed breeds presented for slaughter in three different Egyptian governorates using BLV-CoCoMo-qPCR-2 targeting BLV LTRs and a nested-PCR technique targeting *env-gp51* gene. The BLV-CoCoMo-qPCR-2 data revealed a BLV infection rate of 28.0% (47/168) among the tested beef cattle. However, the nested PCR results revealed a different rate of 25.0% (42/168). Notably, the BLV infection rate among beef cattle in this study is similar to that among dairy cattle in our previous study (25.5%) [11]. Secondly, we used phylogenetic analysis of the partial *env-gp51* gene sequence of the BLV strains isolated from the tested animals to reveal the presence of six genetically distinct strains (b, e, f, g, x, and z) of BLV circulating among Egyptian beef cattle. These strains were clustered into two genotypes of BLV, genotype-1 and genotype-4, among the 11 genotypes circulating elsewhere. The four other strains, e, f, g, and z, had different sequences, compared to those found in the dairy cattle. However, the two strains, b

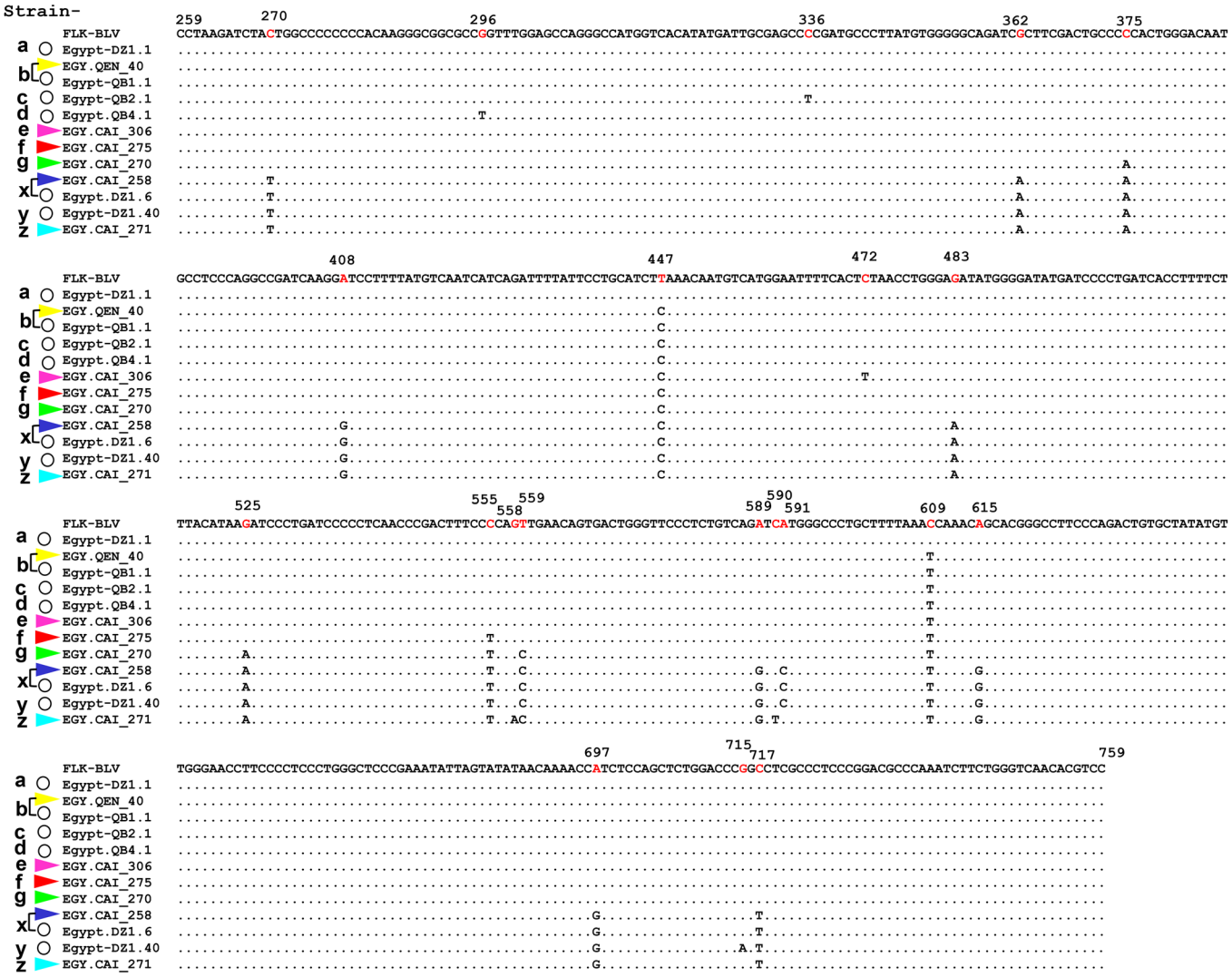
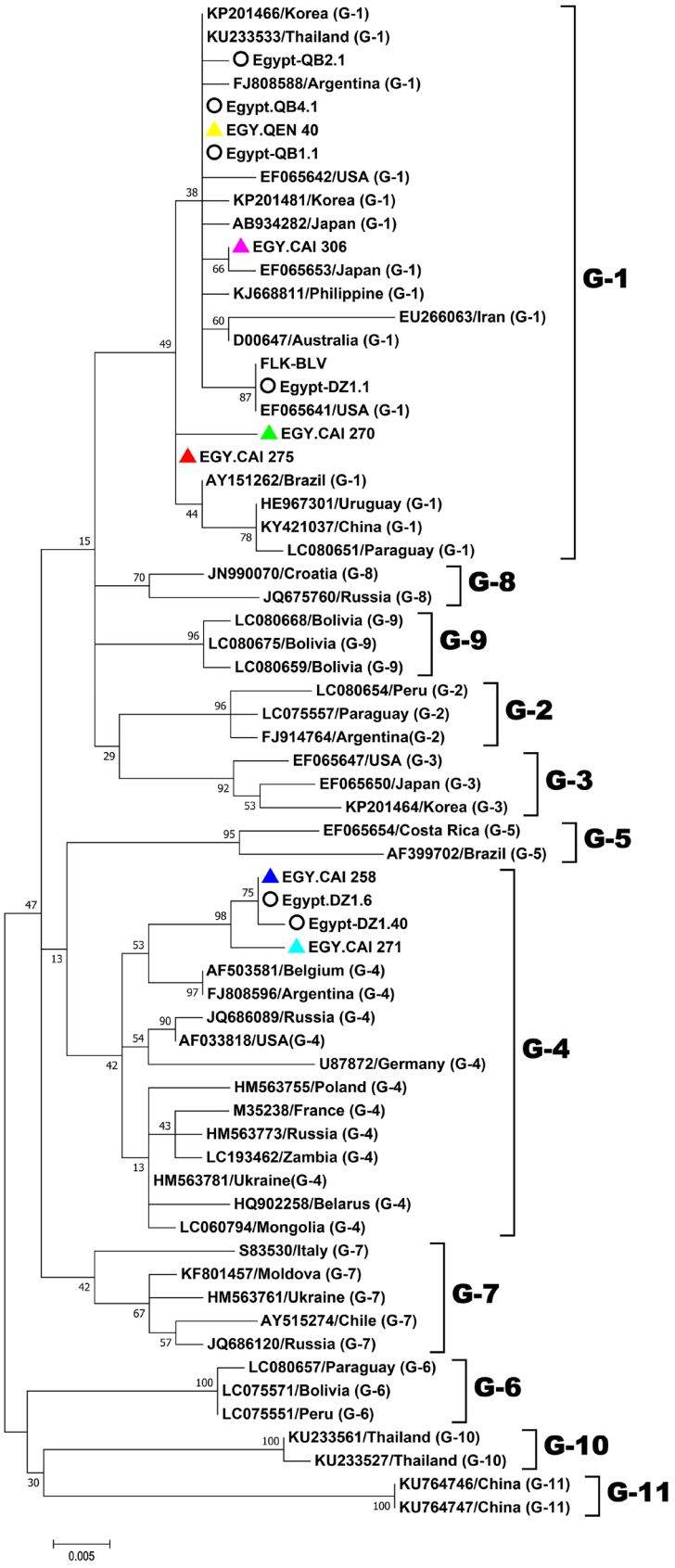


Fig. 2. Alignment of the nucleotide sequences of the 501 bp nucleotide of the bovine leukemia virus (BLV) *env*-gp51 gene from 10 genetically distinct strains circulating in beef cattle (filled triangles) and dairy cattle (opened circles) in Egypt. Alignment was performed for the selected 12 typical Egyptian BLV isolate representatives for the 10 genetically distinct strains circulating in Egyptian cattle (a-g, x, y, and z). The six typical Egyptian beef BLV isolates are indicated by yellow, pink, red, green, blue, and fluorescent filled triangles, respectively. Egyptian BLV isolates are indicated by the country name abbreviation (Egy), farm ID and sample ID. The nucleotide sequence alignment showing the nucleotide substitutions are indicated using numbers and indicated by red on the sequences. Dots represent the pBLV-FLK complete genome (GenBank accession number LC164083) which was used as a reference.

and x, were shared by both dairy and beef cattle in Egypt. Importantly, 20 strains of the 42 isolates identified in the beef cattle in present study, and 38 strains of the 42 isolates identified in dairy cows in the previous study, were classified as the same BLV strain (x) with genotype-4. This indicates that the x strain is widely spread in cattle in Egypt.

The nested-PCR data indicated a lower rate of BLV infections among tested samples than did the BLV-CoCoMo-qPCR-2 data. Only 42 out of 47 samples were positive for BLV provirus, as determined by both nested PCR and BLV-CoCoMo-qPCR-2, but the remaining 5 out of 47 samples were positive by only BLV-CoCoMo-qPCR-2. This could be due to the difference in the targeting genes. BLV-CoCoMo-qPCR-2 can detect even one copy of integrated provirus in the host genome and has been demonstrated to have 100% specificity and low risk of false results [41]. The BLV infection rate found in the present study (28.0%) was higher than that reported in other previous studies in Egypt. In 2012, one group reported an infection rate of 15.83% among cattle, using the agar gel immunodiffusion method and ELISA to detect antibodies against BLV in the blood and milk of foreign breeds of cattle in the Kafr El Sheikh, Alexandria and Menofia governorates [20]. Furthermore, in 2018, a study for screening antibodies against BLV by ELISA revealed an infection rate 20.8% in Kafr El Sheikh, Qalyubia and Menofia [38]. The higher infection rate in this study may indicate that the prevalence of BLV in Egypt has increased in the last few years. However, it could be due to the differences in the methods of BLV detection [14]. The higher prevalence of BLV in beef cattle compared to dairy cattle may be attributed to poor management and the absence of control programs in beef cattle [38]. Alternatively, the breed of cattle investigated may have



influenced the results, as breed has an important role in susceptibility and resistance to BLV infection, due to a variety of genetic and immunological factors [21, 40, 42].

High variation in the BLV prevalence rate existed between the three governorates, Cairo, Fayoum, and Qena; this may be due to geographical, climatic, or farming system causes [24]. The climate and humidity of the Cairo governorate and surrounding area provide good conditions for blood-sucking arthropods, meaning there may be a high risk of within-herd transmission, and could also have an important role in the transmission of BLV between farms [17]. Horizontal transmission of BLV is a major issue, as poor management conditions and intensive farming systems with high numbers of animals allow for direct physical contact [38, 45]. Furthermore, in intensive farming systems in Egypt, farmers often only contact veterinarians in case of emergencies. The use of the same needle and gloves for the treatment or vaccination of multiple individuals can have an important role in the iatrogenic transmission of BLV. Furthermore, BLV has been detected in the saliva, milk, and nasal secretions of cattle [29, 31, 44, 47]. However, more comprehensive studies are needed to confirm the mechanism of BLV transmission through secretions.

The analysis of phylogenetic trees indicated that the beef cattle in our study were infected with six distinct strains of BLV; b, e, f, and g, which belong to the BLV genotype-1; and x and z that belong to genotype-4. Of the samples from the Cairo governorate, 18/39 and 21/39 were infected with BLV genotype-1 and genotype- 4, respectively. However, 1/1 and 2/2 samples from the Fayoum and Qena governorates were infected with genotype-1, respectively. BLV genotype-1,

Fig. 3. Maximum likelihood (ML) phylogenetic tree based on the 420 bp nucleotide of the bovine leukemia virus (BLV) *env*-gp51 gene of the 12 selected typical BLV isolates representing the six strains circulating in Egyptian beef cattle (filled triangles), the six strains isolated from Egyptian dairy cattle (opened circles) and the 53 BLV sequences which represent 11 BLV genotypes derived from viruses isolated worldwide. The six typical BLV isolates from Egyptian beef cattle are indicated by yellow, pink, red, green, blue, and fluorescent filled triangles, respectively. The corresponding GenBank accession numbers are: LC553737 (EGY. QEN 40), LC553741 (EGY. CAI 306), LC553755 (EGY. CAI 275), LC553757 (EGY. CAI 270), LC553768 (EGY. CAI 258), and LC553778 (EGY. CAI 271). Nucleotide sequences were obtained from the GenBank nucleotide sequence database. Sequences are labelled with their accession numbers and countries of origin. The genotypes (G1-G11) are indicated by numbers to the right of the figure. One thousand replications were performed to calculate the bootstrap values indicated on the tree. The bar in the lower portion of the figure denotes distance.

which is prevalent in many countries around the world, including the USA, Costa Rica, Argentina, Uruguay, Brazil, Japan, Korea, Taiwan, Iran, Australia, and Germany; and genotype 4, which is more prevalent in European and South American countries such as Belgium, Poland, Russia, Argentina, and Chile [13, 24, 37]. Both genotypes-1 and -4 were found at the same rate, of 21 samples for each genotype. Egypt imports live cattle for beef and dairy production from many countries all over the world, including South American countries such as Brazil and Paraguay; European countries such as Germany, France and the Netherlands; and the USA [1]. Therefore, transmission of BLV from the endemic countries through livestock importation could be a major source of BLV infection in Egypt [20, 38]. Phylogenetic analysis of the different BLV strains demonstrated that cattle from the Cairo governorate had the highest level of similarity and identity in both genotype-1 and genotype-4, as indicated by strain b and strain x, respectively. This could be due to the intensive farming system that may increase the chance of BLV transmission within the farm [38, 45].

In conclusion, this study was the first to report the prevalence and molecular characteristics of BLV among Egyptian beef cattle. BLV was detected in beef cattle in three governorates in Egypt. Our data demonstrated a higher prevalence of BLV in Egypt compared to the seroprevalence previously reported. The genetic variability of the identified isolates revealed the presence of six strains of BLV, belonging to genotype-1 and -4, among beef cattle in Egypt. These results demonstrated the urgent need for control and preventive measures in Egyptian beef cattle farms.

CONFLICTS OF INTEREST. The authors declare no conflicts of interest.

ACKNOWLEDGMENTS. We thank the veterinarians and our collaborators for kindly assisting with sampling from slaughter houses in Egypt. We are grateful to members of the Viral Infectious Diseases Field, RIKEN, for their technical assistance, help, and suggestions, and Support Unit at the Bio-material Analysis, RIKEN BSI Research Resources Center, for help with sequence analysis. This work was supported by a Grant-in-Aid for Scientific Research (A) from the Japan Society for the Promotion of Science (JSPS) (Grant no. 16H02590) and by a grant from Embassy of Egypt Culture, Education and Science Bureau.

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